
THE UNIVERSITY *of* EDINBURGH

# Causes and consequences of asynchronous hatching in the burying beetle *Nicrophorus vespilloides*

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# Declarations

This dissertation is submitted in accordance with the requirements for a Doctorate of Philosophy by the School of Biological Sciences at The University of Edinburgh. I declare that I have written this thesis under the guidance of my supervisor. I conducted all experimental work with help as below. All other work was my own.

Chapter 4: The data were collected by Kirsten Henderson under my supervision. I performed all analyses presented here and wrote the chapter with guidance from Per Smiseth.

Chapter 5: The data were collected with assistance from Laura Crichton. Ed Ivimey-Cook helped to rear the beetles used in this experiment. I performed the analyses and wrote the chapter in collaboration with Jacob Moorad and Per Smiseth.

The work included in this thesis has not been submitted for any other degree or professional qualification.

Lucy Ford

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# Lay Summary

The species studied in this thesis is the burying beetle, an insect that cares for its young (larvae) after they hatch. Female burying beetles lay their eggs around a small carcass, but they do not lay all of their eggs at the same time. This means that the eggs hatch over a couple of days (termed asynchronous hatching). The growth and survival of the larvae is affected by when they hatch. The larvae grow very quickly, so by the time the last larvae hatch the older larvae are already a lot bigger than them. I explore the consequences of asynchronous hatching for the larvae and their parents. There are many potential causes of asynchronous hatching and I address four here. 1) Females might lay more asynchronously to make males provide more care to the larvae. I find that males do stay longer when the larvae hatch over a longer period of time, but this also causes more of the larvae to die and those that survive are smaller so it is not worthwhile for females to manipulate males by laying more asynchronously. 2) Starting to lay sooner after encountering the carcass might cause females to lay more asynchronously. I find that females do lay over a longer period of time when they start to lay sooner, but they do not lay earlier when the quantity or quality of the carcass they are breeding on is lower. 3) Inbred females (females whose parents were related) might be of poorer quality and this might cause them to lay more asynchronously. I find the opposite; inbred females appear to lay their eggs in a way that would improve the survival of the larvae. 4) Older females may produce laying patterns that are less beneficial to larval growth and survival than young females. Instead, I find that there is less variation in laying times in clutches produced by older females compared to those produced by younger females. There are many potential causes of asynchronous hatching that can interact to determine the hatching pattern, with important consequences for offspring growth and survival.

# Abstract

In this thesis, I explore the causes and consequences of asynchronous hatching (when the offspring form a single reproductive event hatch over an extended period of time) in the burying beetle *Nicrophorus vespilloides*, an insect with biparental care. Hatching asynchrony can be influenced by both intrinsic and extrinsic factors and may be adaptive or a consequence of constraints on egg laying. The four potential causes of asynchronous hatching I focus on here are 1) sexual conflict over parental care, 2) physiological constraints on completing reproduction quickly, 3) inbreeding and 4) ageing. The sexual conflict hypothesis for the evolution of asynchronous hatching suggests that females adjust hatching patterns in order to increase male parental effort relative to female effort. As predicted, I found that males provided care for longer to asynchronous broods whereas the opposite was true of females. However, I did not find any benefit to females of reducing their duration of care in terms of their mass change or lifespan, and I found substantial negative effects of hatching asynchrony on offspring fitness. The hurry-up hypothesis suggests that completing reproduction quickly is beneficial when the quantity or quality of breeding resources declines over time and this may lead to asynchronous hatching if there are physiological constraints on laying. My results suggest that, although asynchronous hatching might emerge as a by-product of parents attempting to complete reproduction sooner, there is no evidence that females attempt to do so sooner under conditions where this would be favourable. Inbred females may be constrained in their laying patterns if they are of poorer quality, which might cause them to lay more asynchronously. Conversely, I found that inbred mothers produced clutches where egg laying was less skewed towards the early part of laying, improving larval survival. Inbred females may facultatively adjust their laying patterns to compensate for the detrimental effects of maternal inbreeding on offspring. If selection on laying patterns is weaker at older ages, females breeding at older ages might lay more asynchronously. Instead, I found that females breeding at older ages produce clutches with a lower within-brood variance in laying times and that were less positively skewed. Age-related reductions in clutch size were also associated with reduced within-clutch variance in laying times and a less positively skewed distribution of laying times. Thus the indirect effect of female age mediated through clutch size reinforces the direct effect of female age on the variance and skew in laying times. I conclude that asynchronous hatching in *N. vespilloides* is unlikely to be explained by a single cause.

# Chapter 1

# General Introduction

## 1.1 *What is asynchronous hatching?*

Asynchronous hatching is when the offspring from a single reproductive event hatch (or are born) over an extended period of time. Asynchronous hatching has been studied primarily in birds (Lack, 1947), but also occurs in other taxa, including reptiles (While *et al*., 2007), amphibians (Ryan and Plague, 2004), elasmobranchs (Gilmore, 1993) and insects (Nalepa, 1998) including the burying beetle *Nicrophorus vespilloides* Herbst (Smiseth *et al*., 2006). In birds, hatching asynchrony is defined as incubation before clutch completion because hatching will be synchronous if the onset of incubation occurs after the clutch has been completed, and asynchronous if incubation begins before the last egg has been laid (Clark and Wilson, 1981). However, this definition is not applicable nonavian taxa, which do not have incubation. Comparable hatching asynchrony occurs in many such taxa and hatching asynchrony can have similar consequences as it does in birds, despite arising through different mechanisms.

## 1.2 *The study system Nicrophorus vespilloides*

The study species I focus on is the burying beetle *Nicrophorus vespilloides* (Herbst). Burying beetles provide parental care to their larvae (Pukowski, 1933), which improves offspring survival (Eggert *et al*., 1998). Care may be biparental, maternal or paternal (Bartlett, 1988). These beetles bury a small vertebrate carcass and females lay their eggs in the surrounding soil. Females have two ovaries with around 12 ovarioles, each containing 2 or 3 oocytes (Müller unpubl. in Smiseth *et al*., 2008). They adjust the number of eggs they lay depending on the size of the carcass, and can lay more than 40 eggs in a clutch (Bartlett and Ashworth, 1988). In *N. vespilloides*, hatching often occurs asynchronously (Müller and Eggert, 1990). Shortly after hatching, the larvae crawl to the carcass. Parents prepare the carcass by producing exudates to reduce microbial growth, removing hair and making an opening where the larvae can gain access to feed for themselves. Parents also directly feed the larvae by regurgitating carrion (Smiseth *et al*., 2003), which increases larval growth and accelerates development (Lock *et al*., 2004). The larvae grow rapidly until they disperse away from the carcass into the soil. The mean brood size at dispersal is 21 larvae (Smiseth and Moore, 2002). The larvae then pupate underground and eventually eclose as adults. They must then seek a carcass that is suitable for breeding. There is strong competition for carcasses (Trumbo, 1990a). Sometimes multiple females breed on the same carcass (Komdeur, *et al*., 2013)

where they may each care for their own larvae or become brood parasites (Müller *et al*., 1990a). Offspring on 57% of carcasses in the field were from more than one female (Müller *et al*., 2007). Additionally, multiple paternity within broods is common (Eggert, 1992; House *et al*., 2008).

### 1.3 *Why is asynchronous hatching important?*

Hatching asynchrony can have profound consequences on offspring growth and survival. Some studies have found that offspring survival is increased by hatching asynchrony (Hahn, 1981; Magrath, 1989), while other studies indicate that greater synchrony is favourable (Hébert, 1993). Hatching asynchrony also affects offspring survival in *N. vespilloides* (Smiseth *et al*., 2008; Smiseth and Morgan, 2009). The duration of time over which the larvae hatch affects the number of larvae reaching dispersal; a greater number of larvae survive in experimentally generated broods that simulate hatching over 12 hours compared to 24 hours or all larvae arriving at the carcass simultaneously (Smiseth *et al*., 2008) and survival is decreased when larvae are introduced over 48h compared to 0 or 24h (Smiseth and Morgan, 2009). Although asynchronous broods may produce fewer fledglings in birds, the higher quality of the surviving offspring may compensate for this (Slagsvold, 1986). For example, mean body mass is sometimes higher in asynchronous broods (Amundsen and Slagsvold, 1991). The costs to parents of providing parental care may also differ between producing synchronous or asynchronous broods (Hussell, 1972). Therefore, the optimal degree of asynchrony may depend on multiple factors and is likely to differ depending on the species and ecological context.

The effects of hatching asynchrony on offspring can be mediated through interactions between the siblings. Asynchronous hatching creates a size hierarchy among the offspring (Lack, 1954; Mock and Parker, 1997), which establishes competitive asymmetries within the brood (Mock, 1984; Magrath, 1990; Stoleson and Beissinger, 1995; Mock and Parker, 1997; Parker *et al*., 1989). Offspring that hatch later are competitively disadvantaged compared to their siblings that hatched earlier (Howe, 1976; O'Connor, 1978; Clark and Wilson, 1981; Mock, 1984). Thus, the distribution of hatching times within a brood can affect offspring growth and survival (Hahn, 1981; Magrath, 1989; Hébert, 1993; Smiseth *et al*., 2008; Smiseth and Morgan, 2009). Size hierarchies resulting from asynchronous hatching can have a greater effect on within-clutch variation in performance of offspring than egg size (Maddox and Weatherhead, 2008; Bitton *et al*., 2006). The significant effect of

hatching asynchrony on sibling competition has led to this effect being proposed as a potential reason for the evolution of asynchronous hatching. The sibling rivalry hypothesis (Hahn, 1981) suggests that the stable dominance hierarchy generated by size differences between offspring caused by asynchronous hatching reduces the amount of energy wasted through fighting between siblings and therefore uses parental resources more efficiently. As a result, total offspring energy expenditure is predicted to be higher for synchronous broods than asynchronous broods due to additional energy expended on competition, which is detrimental to offspring growth and survival (Clark and Wilson, 1981). Parents may be expected to expend more energy provisioning more synchronous broods to compensate for the energy wasted through offspring competition. Asymmetric sibling competition also occurs in burying beetles, where larvae compete for access to the parent to receive regurgitated food. Late-hatched offspring gain less access to the mouthparts of the parent (Andrews and Smiseth, 2013) and therefore experience reduced growth despite spending more time begging (Smiseth *et al*., 2007a) and are less likely to survive to independence than early-hatched larvae (*N. quadripunctatus*, Takata *et al*., 2013).

### 1.4 *Current understanding of causes hatching asynchrony*

There are over 17 hypotheses to explain the occurrence of asynchronous hatching in birds (reviewed in Stoleson and Beissinger, 1995; Stenning, 1996), many of which may also apply to burying beetles. These include the brood reduction hypothesis (Lack, 1954), the sibling rivalry hypothesis (Hahn, 1981), the peak load reduction hypothesis (Hussell, 1972), the insurance hypothesis (Stinson, 1979), icebox hypothesis (Alexander, 1974), the sexual conflict (exploitation of mate) hypothesis (Slagsvold and Lifjeld, 1989; Slagsvold *et al*., 1995), the limited breeding opportunity hypothesis (Beissinger and Waltman, 1991) and the hurry-up hypothesis (Hussell, 1972; Clark and Wilson, 1981; Slagsvold, 1986). Hatching asynchrony may also be influenced by brood parasitism, the frequency of multiple paternity and genetic constraints. It has been suggested that asynchrony may serve as a bet-hedging strategy to reduce the variability in the likely number of offspring recruited each year compared to synchronous hatching (offspring quality assurance hypothesis, Amundsen and Slagsvold, 1991, 1998; offspring diversity hypothesis, Laaksonen, 2004). Currently none of these hypotheses are universally accepted as an explanation for hatching asynchrony in all circumstances; it is likely that there are

multiple factors causing asynchrony in different situations (Stoleson and Beissinger, 1995; Stenning, 1996).

In burying beetles, parents do not incubate the eggs and instead hatching patterns are determined by laying patterns (Smiseth *et al*., 2006). This allows hypotheses relating to optimal timing of incubation, which may result in asynchronous hatching as a by-product in birds, to be separated from those relating to adaptive hatching patterns (Smiseth *et al*., 2006). The mean hatching spread (the period of time between the first and last offspring hatching) is 30 hours (Smiseth *et al*., 2006) and can range between 8 and 56 hours, depending on the size of the carcass (Müller and Eggert, 1990). The larvae reach nutritional independence at 72 hours (Smiseth *et al*., 2003) and disperse from the carcass synchronously around 6 days after hatching under laboratory conditions (Smiseth *et al*., 2006); therefore the hatching spread is considerable relative to the overall time spent on the carcass. The majority of the eggs are laid towards the beginning of the laying period (Müller, 1987; Smiseth *et al*. 2006), and as a result hatching is positively skewed towards the start of the hatching period, which is also the case for most birds (Magrath, 1990). It is possible that there is a heritable basis to hatching asynchrony in *N. vespilloides* (Smiseth *et al*., 2008). The only hypothesis for the evolution of asynchronous hatching that had been explicitly tested in *Nicrophorus* prior to this project is the peak load reduction hypothesis. This hypothesis suggests that parents can avoid the peak in food demand for each offspring coinciding through asynchronous hatching and thus the parents can save energy (Hussell, 1972; Mock and Schwagmeyer, 1990). The hypothesis was not supported because the peak in female food provisioning did not change amplitude or timing depending on the degree of hatching asynchrony of the brood, despite the decrease in peak offspring demand with greater asynchrony (Smiseth and Morgan, 2009).

### 1.5 *Adaptive reasons for hatching asynchrony*

The sexual conflict hypothesis (Slagsvold and Lifjeld, 1989) proposes that the resolution of sexual conflict over parental care could be mediated through asynchronous hatching. I test this hypothesis in Chapter 2. Sexual conflict can arise over how much care each parent should provide and each parent is expected to minimize its costs of care by shifting as much of the workload as possible over to its partner (Trivers, 1972). In *N. vespilloides*, both parents are present on 59% on carcasses in the field, but the male had left the carcass in 39% of cases one day

after burial (Eggert, 1992). Females are capable of raising the same size and number of larvae with or without male assistance in the lab (Smiseth *et al*., 2005) and in the field (Müller *et al*., 1998). However, there may be some costs to females of raising the brood alone which become evident in future reproductive bouts (Jenkins *et al*., 2000). The likelihood that a parent beetle will desert the brood depends on clutch size and the individual's potential future reproductive output (Ward *et al*., 2009). Sexual conflict occurs over parental care in *N. vespilloides* because males potentially have a greater lifetime reproductive success than females through polygyny (Trumbo and Eggert, 1994). Males release pheromones to attract additional females which they inseminate (Pukowski, 1933; Müller and Eggert, 1987; Eggert and Müller, 1989; Eggert, 1992). The probability of future reproduction could also be influenced by the likelihood of securing a carcass. Females may be assisted to find a carcass by males releasing pheromones, but if there is already a resident pair the female may be forced to seek another carcass. Male burying beetles can also produce offspring without acquiring a carcass by mating with any females they encounter. If an inseminated female later finds a carcass she will use the male's sperm to fertilise her eggs. This sex difference in the probability of finding a carcass to breed on could explain the difference in residency times (Eggert and Müller, 1997). Although males usually desert the brood sooner than females, they may alter their behaviour in response to cues regarding offspring. Females may therefore benefit from adjusting their laying patterns in order to manipulate males to increase their duration of care, allowing females to reduce their own contribution.

The most prominent hypotheses for the evolution of asynchronous hatching in birds are probably the brood reduction hypothesis and the insurance hypothesis, which were widely accepted for many years (Stenning, 1996). The brood reduction hypothesis suggests that parents deliberately create size differences between offspring through asynchronous hatching so that brood size can be adjusted to match resource availability, which cannot be predicted when the eggs are laid (Lack, 1954; Ricklefs, 1968). All chicks survive when conditions are favourable and smaller offspring are out-competed by their siblings and starve when resources are scarce, increasing the survival of the first-hatched offspring. In support of this hypothesis, the probability that the remaining young survive is higher after the brood is reduced compared to the probability of each offspring surviving if no brood reduction has occurred (O'Connor, 1978). Parents may contribute to brood reduction by preferentially feeding the older, larger offspring when food is scarce. For example,

White-winged choughs preferentially feed larger chicks when food is scarce and favour smaller chicks when supplemental food is provided (Boland *et al*., 1997), and American Kestrels hatch more synchronously when food is plentiful (Wiebe and Bortolotti, 1994). However, a number of studies have found evidence that does not support the hypothesis (e.g. Stamps *et al*., 1985; Hillström and Olsson, 1994). The brood reduction hypothesis was proposed as an explanation for mortality of the smallest offspring and does not necessarily imply that the smallest offspring will always be most likely to starve when conditions are unfavourable if hatching occurs asynchronously. Brood reduction can still occur if hatching is synchronous as some offspring will inevitably die if there are insufficient resources, but asynchrony allows smaller offspring to die sooner than would occur in a synchronous brood and therefore parents waste fewer resources caring for offspring which will not survive (Mock and Parker, 1986).

Brood reduction can be implemented through infanticide (including indirectly due to parental neglect) or siblicide (including indirectly due to competition and aggression), with a lower threshold disparity between brood size and resource availability required for siblicide compared to infanticide (O'Connor, 1978). The disparity between the number of offspring and the amount of food for the offspring is generated by uncertainty in food supply in birds, whereas in burying beetles there is uncertainty in demand because they cannot accurately predict how many eggs will hatch. In contrast to birds, the food resource required for development of burying beetle larvae is already present when the eggs are laid (Eggert and Müller, 1997) and brood reduction in *Nicrophorus* occurs through filial cannibalism (Bartlett, 1987). Larvae are more likely to be eaten by the parent when begging (Andrews and Smiseth, 2013) and younger larvae spend a greater amount of time begging than senior larvae (Smiseth *et al*., 2007a). Thus it is likely more young larvae will be eaten, as was found by Takata *et al*. (2013) in *N. quadripunctatus*. Parents increase the mortality risk of late-hatched larvae by exacerbating sibling competition (Smiseth *et al*., 2007b). This is in accordance with the brood reduction hypothesis which suggests that the greatest risk of mortality is faced by younger offspring which have received less parental investment and would require the most future investment in order to survive. Testing this hypothesis would require demonstrating whether mortality of some larvae ultimately increased the number and size of surviving larvae. I experimentally test how the degree of hatching asynchrony affects larval number and mass in Chapter 2. However, any differences observed cannot be

directly attributed to more efficient removal of late-hatched larvae in asynchronous broods, and it is not possible to experimentally prevent larval mortality to compare broods with and without reduction. Despite the impracticality of testing this hypothesis explicitly, the concepts that brood reduction can increase growth and survival of the remaining offspring and that asynchronous hatching increases the efficiency of brood reduction are relevant in the context of other hypotheses for the evolution of asynchronous hatching where offspring mortality is involved.

The insurance hypothesis (Stinson, 1979) suggests that females lay more eggs than they can rear in case some eggs do not hatch. The later-hatched offspring do not normally survive unless earlier offspring fail to hatch or die soon after hatching. If all of the early-laid eggs hatch, and there are insufficient resources for later offspring to also survive (Taylor and Perrin, 2008), asynchronous hatching may allow relatively efficient removal of the last-laid offspring (Forbes, 1990). Burying beetles lay a greater number of eggs than can be supported by the carcass if they all develop into larvae. Some of these eggs may fail to hatch and therefore additional eggs may serve as insurance to replace any offspring that do not survive (Bartlett, 1987). If a greater number of larvae arrive at the carcass than can be supported, excess larvae are removed by filial cannibalism. A greater proportion of larvae are predicted to survive in asynchronous broods than synchronous broods of the same initial size because in asynchronous broods excess offspring are removed at a young age so fewer resources are wasted on offspring which will not survive. In contrast to the brood reduction hypothesis, the insurance hypothesis predicts that the mortality of the additional insurance offspring depends only on the survival of the early-hatched (or early-laid) offspring and therefore is not directly dependent on the availability of food resources. Testing this hypothesis would require experimentally altering the degree of hatching asynchrony and the mortality of the first-hatched offspring and assessing how this affects the growth and survival of the remaining larvae. It is difficult to determine whether mortality of the early-hatched larvae increases survival of the later-hatched larvae because individual larvae cannot be distinguished. It is possible to mark larvae by cutting their legs but this could affect their ability to beg and could increase mortality. As a result of this unfeasibility I do not test the insurance hypothesis, however, given the potential unpredictability of egg viability and low hatching success in *N. vespilloides* (Bartlett, 1987) this hypothesis is likely to be relevant in conjunction with other causes of hatching asynchrony. For example, carcass decomposition (Chapter 3) reduces egg viability

(Jacobs *et al*., 2014) and early larval survival (Rozen *et al*., 2008) resulting in unpredictability regarding whether the carcass can meet offspring demand. The brood reduction hypothesis and/or insurance hypothesis may then take effect.

### 1.6 *Nonadaptive reasons for hatching asynchrony*

Some of the hypotheses for the evolution of asynchronous hatching relate to selection or constraints on the timing of incubation that lead to asynchronous hatching, rather than selection on hatching patterns directly. These hypotheses include the egg viability hypothesis (Arnold *et al*., 1987), egg protection hypothesis (Beissinger *et al*., 1998), nest failure hypothesis (Worth, 1940; Clark and Wilson, 1981) predation hypothesis (Clark and Wilson, 1981) and hurry-up hypothesis (Hussell, 1972; Clark and Wilson, 1981; Slagsvold, 1986). Aside from selection for incubation patterns incidentally influencing hatching asynchrony in birds, the degree of synchrony could also be physiologically constrained. For example, asynchronous hatching could be influenced by clutch size or the delay until laying commences. This could be the case for burying beetles as well as birds (Eggert *et al*., 2008). Because burying beetles do not mature their oocytes until they secure a carcass for breeding (Wilson and Knollenberg, 1984), physiological constraints may lead to greater laying asynchrony when females begin laying soon after encountering the carcass if there has not been sufficient time for maturation of all of the ovarioles. Many factors could potentially influence the timing of oviposition. For example, there is a longer delay between encountering a carcass and the onset of oviposition in co-breeding females compared to single females (Eggert and Müller, 2000). The nutritional condition of a female may dictate the delay until oviposition (Trumbo *et al*., 1995; Steiger, 2013), the potential duration of the oviposition period (Eggert and Müller, 2011) or laying spread (Steiger, 2013). Other factors that influence body condition could also constrain laying, including female size, age or inbreeding status. Hatching asynchrony may also be influenced indirectly by clutch size if females take longer to lay more eggs. Indeed, eggs hatch over a longer period of time and hatching is more strongly positively skewed towards the start of the hatching period in larger clutches (Smiseth *et al*., 2008). Clutch size in turn can be affected by female condition (Steiger *et al*., 2007; Steiger, 2013) and body size (Bartlett and Ashworth, 1988; Steiger, 2013).

The main hypotheses for the evolution of asynchronous hatching as a nonadaptive by-product are the hurry-up hypothesis (Hussell, 1972; Clark and

Wilson, 1981; Slagsvold, 1986) and the limited breeding opportunity hypothesis (Beissinger and Waltman, 1991). The hurry-up hypothesis suggests that completion of reproduction as soon as possible is favoured when the quantity or quality of food resources declines over time (Hussell, 1972; Clark and Wilson, 1981; Slagsvold, 1986). I test this hypothesis in Chapter 3. In birds, parents can reduce the time between the start of laying and the first offspring hatching by commencing incubation before the clutch is completed, resulting in asynchronous hatching of the clutch (Clark and Wilson, 1981). Thus, it has been suggested that under some circumstances asynchronous hatching may occur as a by-product of parents attempting to complete reproduction more rapidly. In some bird species, young from early broods have greater chances of survival than those from later broods (e.g. Perrins, 1965). This would favour early onset of incubation, and therefore more asynchronous hatching as the season progresses (Slagsvold, 1986). Similarly, Scott and Traniello (1990) found that the total brood mass of the burying beetle *N. orbicollis* was greater for broods that were started earlier in the breeding season, possibly partly due to the competition with flies being less intense. In addition to the potential benefits of breeding early in the season, burying beetles may be selected to lay as soon as possible after finding a suitable carcass because the value of the carcass for reproduction decreases over time as it decomposes, as the quantity and quality of food remaining declines (Müller, 1987). Therefore, beetles arriving at carcasses which have decomposed to a greater extent may attempt to lay sooner. High levels of competition for optimally-sized carcasses may also select for early onset of oviposition upon encountering a suitable carcass because the value of the carcass to other beetles which may attempt a take-over declines rapidly once the resident larvae have begun to consume it (Trumbo, 2006). In addition, larger carcasses are more likely to be usurped by other burying beetles (Trumbo, 1991) which will kill the entire resident brood (Trumbo, 1990a), and it is more difficult to control microbial activity on larger carcasses (Trumbo, 1992). Therefore, carcass size (resource quantity) and decomposition (resource quality) may provide an incentive to accelerate reproduction and this may lead to laying asynchrony due to physiological constraints on laying.

The limited breeding opportunity hypothesis (Beissinger and Waltman, 1991) is based on the premise that because reproductive effort should be high when residual reproductive value is low (Williams, 1966), individuals should be selected to invest more in current reproduction when there is a high level of competition for

reproductive opportunities as they are unlikely to get many subsequent opportunities to breed. In line with this, male burying beetles increase the duration of care they provide when there is a higher perceived population density (Scott, 1998a) and females facing an immune challenge invest more in current reproduction (Reavey *et al*., 2015). Birds laying larger clutches may commence incubation before clutch completion in order to protect the eggs and maintain their viability, resulting in hatching asynchrony (Beissinger and Waltman, 1991). Hatching asynchrony in burying beetles may also be a by-product of producing large clutches. Due to the scarcity of suitable carcasses and the intense competition for these breeding resources (Trumbo, 1990a), females may be selected to lay large clutches when sufficient resources are available in order to make the most of the breeding opportunity. If there are physiological constraints on laying, attempting to lay a large number of eggs could result in asynchronous hatching because laying spread increases with clutch size (Smiseth *et al*., 2008). I do not test this hypothesis explicitly but I test for an effect of clutch size on the degree of hatching asynchrony (Chapters 2, 3 and 5), as well as other potential nonadaptive causes of asynchronous hatching through constraints such as female age (Chapter 5) and inbreeding status (Chapter 4).

### 1.7 *Reducing/exacerbating the consequences of asynchronous hatching*

If asynchronous hatching is nonadaptive, there may be selection for mechanisms that reduce the disparity between offspring caused by hatching asynchrony. Birds are often limited to laying one egg per day and therefore the clutch will not hatch synchronously unless they delay incubation until after clutch completion, which may be undesirable if it reduces egg viability (Arnold *et al*., 1987) or renders the eggs more vulnerable (Beissinger *et al*., 1998). Some bird species reduce the hatching asynchrony generated by laying asynchrony through accelerated development of the last-laid eggs relative to the first-laid eggs when the offspring can hear their siblings or detect other cues (Vince, 1964; 1966; Schwagmeyer *et al*., 1991; Persson and Andersson, 1999; Clark *et al*., 2009; Hadfield *et al*., 2013). Nonavian species also synchronise hatching or emergence using auditory or potentially vibration cues from siblings when the eggs are in proximity (fish Bradbury *et al*., 2005, amphibians Sih and Moore 1993; Warkentin 1995, 2000, lizards, Vitt 1991, and turtles Doody *et al*., 2001, 2012; Spencer *et al*., 2001).

Alternatively, the effects of hatching asynchrony on offspring variation within the brood can be mitigated by altering egg size to counteract the effect of hatching order. For example, hatching asynchrony is unavoidable for Gentoo penguins because it is so cold eggs must be incubated as soon as they are laid, but the second egg is larger to reduce the competitive disadvantage faced by the second chick (Williams and Croxall, 1990). Laying larger second eggs can even reverse hatching order compared to laying order in crested penguins because egg size affects the duration of development (Warham, 1975). Another potential mechanism to counteract the effects of hatching asynchrony is the addition of differing amounts of hormones to the eggs depending on laying order to increase competitive ability and development rate of later offspring (Schwabl, 1996). In birds, the concentration of androgens often varies between eggs based on their position in a brood (Schwabl, 1993) and much of the variation in the pattern of hormone allocation across the clutch can be attributed to maternal genes (Groothuis *et al.*, 2008). Females could therefore be under selection to allocate different amounts of androgens to each offspring depending on its position in the laying order (Muller and Groothuis, 2013). There may be a cost to these mechanisms that reduce hatching asynchrony, such as reduced hatching mass when development is accelerated (Muck and Nager, 2006), or increased energy expenditure (Tobler *et al*., 2007) and decreased immune function (Pierce *et al*., 1966) associated with increasing egg androgens. This may limit the amount of compensation for asynchrony that is beneficial. Parental provisioning behaviour could also mitigate the effects of asynchronous hatching, for example feeding later-hatched offspring more frequently (Gottlander, 1987) or provisioning them with more nutritious prey species (Garcia-Navas *et al*., 2014).

In contrast, if hatching asynchrony is adaptive, females may intensify the effects of hatching asynchrony rather than attempt to counteract them. Species with relatively low hatching asynchrony may attempt to reduce asymmetries between offspring, but in some cases when there are large clutches with extreme asynchrony, mothers may deliberately amplify competitive asymmetries to allow efficient removal of smaller offspring when resources are insufficient to raise the entire brood. In birds, mothers in species with greater hatching asynchrony exacerbate the asymmetric sibling competition caused by hatching asynchrony by reducing the compensatory allocation of testosterone to late chicks (Müller and Groothius 2013). Burying beetles could alter the strength of the effects of hatching

asynchrony on offspring by employing similar mechanisms to those in birds, including accelerated or delayed development of last-laid eggs, increasing or decreasing egg size across the clutch, and addition of hormones to the eggs in differing amounts across the laying order. In *N. vespilloides*, last-laid eggs hatch slightly quicker than first-laid eggs (Smiseth *et al*., 2006), whereas last eggs take longer to hatch in *N. quadripunctatus* (Takata *et al*., 2015). *N. vespilloides* mothers may add hormones such as juvenile hormone to the eggs which stimulates begging (Crook *et al*., 2008), but it is not yet known whether females actively manipulate hormone levels across the laying order. I test for an increase in egg size across the clutch in Chapter 3.

### 1.8 *Methodological information*

I have developed a technique to record laying patterns using flatbed scanners (Canon Canoscan 9000F Mark II, Canon Inc., Tokyo, Japan). I placed females (and males, depending on the experiment) along with a carcass in a clear breeding box (170mm x 120mm x 60mm) containing 1cm of moist compost. Eggs are visible at the bottom of the breeding box, and the visible number of eggs is very similar to the actual clutch size (Pearson's correlation: $r = 0.98$, $n = 21$, $P < 0.001$, Monteith *et al*., 2012). Figure 1.1 shows a scan of one breeding box. If the amount of soil is insufficient the beetles clear it around the carcass and move the soil up the edges of the box, which can result in eggs being laid up the sides where they are not visible. Similarly, if more than 1cm of compost is used, some of the eggs may be laid just below the surface of the soil rather than right at the bottom of the box and so may not be visible on the scans. I scanned the breeding boxes every hour using Vuescan professional edition software (Hamrick Software, Sunny Isles Beach, FL, USA) until the eggs hatched. I set the resolution to 600dpi, which is sufficient for measuring eggs. The beetles did not appear to be disturbed by the light of the scanners as they continued to prepare the carcass while the box was scanned and had comparable breeding success to the other beetles in the lab. This technique allows up to 20 clutches to be monitored simultaneously without continuous presence in the lab; once the scanners are set up the images are recorded automatically, allowing much greater sample sizes to be obtained than previous methods. Using scanners also has the advantage of being less invasive because it does not require disturbing females to search through the soil to find eggs. This avoids potential damage to the eggs that could be sustained when they are transferred to a petri dish and also

avoids the risk of dehydration of eggs and larvae. Because the scanner technique allows eggs to remain *in situ* with the carcass, larval development can continue as usual in the presence of the carcass and adults, allowing data to be collected on larval survival and mass at dispersal along with laying patterns of each clutch.

I counted the number of eggs on each scan. First, I removed the initial scans with no eggs to determine the time until the first egg was laid after the female encountered the carcass. I then subtracted the number of eggs on the previous scan from the number on each scan to determine the number of new eggs laid each hour. The sum of these is the total clutch size. This is potentially a slight overestimate because if an egg is visible on a scan then is not visible on the next scan but is visible again on subsequent scans it will be counted as two eggs. However, this is more accurate than using the maximum number of eggs visible on any given scan which can considerably underestimate clutch size.

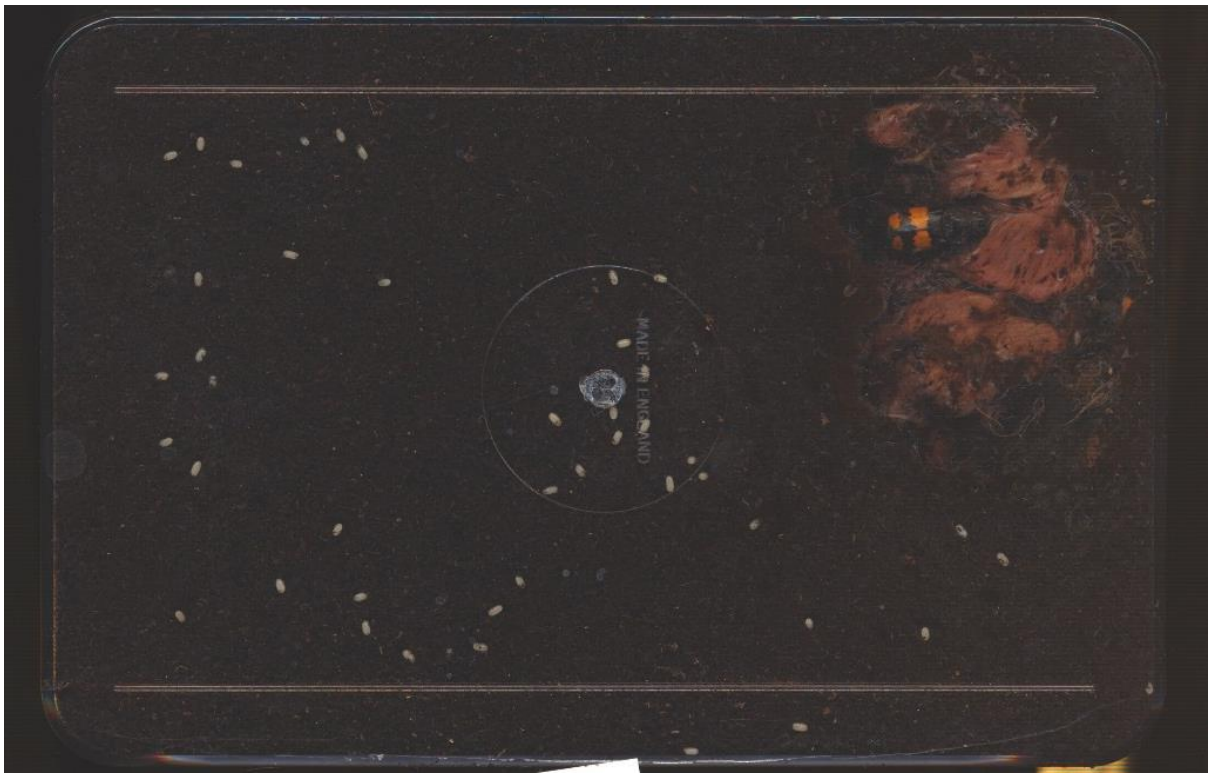


Figure 1.1: Scan of a breeding box. The white dots are eggs. The parents can be seen preparing the carcass in the top right corner.

Hatching asynchrony in birds has generally been measured in terms of when incubation starts in the laying sequence (Clark and Wilson, 1981), which is not applicable in species without incubation, or hatching spread (Stoleson and Beissinger, 1995) which is the time between the first and last chick hatching. For most chapters I used two metrics of hatching asynchrony; laying spread (the time between the first and last egg being laid) and laying skew (the degree to which laying is skewed towards the start of the laying period). Laying spread provides an indication of the degree of hatching asynchrony through the duration of laying but only takes account of the extremes of the distribution of laying times. Measuring laying skew complements the laying spread because it gives an indication of the shape of the distribution of laying times by accounting for the laying time of each egg in relation to the middle of the laying period. However, it accounts for the timing of laying of eggs relative to each other rather than reflecting the absolute duration of laying, and can be independent of laying spread (Smiseth *et al*., 2008; Takata *et al*., 2013). Taken together, laying spread and laying skew allow informative quantification of the degree of hatching asynchrony. I calculated a laying skew index (based on the hatching skew index of Smiseth *et al*., 2008) for each brood using the following formula: $\Sigma((t_i - t_m)/t_m)p_i$, where $p_i$ is the proportion of the total clutch laid each hour, $t_i$ is the time interval starting from the initiation of oviposition and $t_m$ is the middle of the laying period. Values between -1 and 0 indicate that most eggs are laid during the first half of the laying period (positively skewed/ right-skewed distribution of laying times), 0 represents a symmetrical distribution of laying times and values between 0 and 1 indicate that most eggs are laid during the last half of the laying period (negatively skewed/ left-skewed distribution of laying times) (Figure 1.2). Values further from zero reflect more asymmetrical (more strongly skewed) distributions of laying times. Laying skew index is not necessarily related to the timing of initiation of oviposition after the female encounters the carcass because it is calculated relative to the start of the laying period for each clutch rather than the absolute timing of laying.

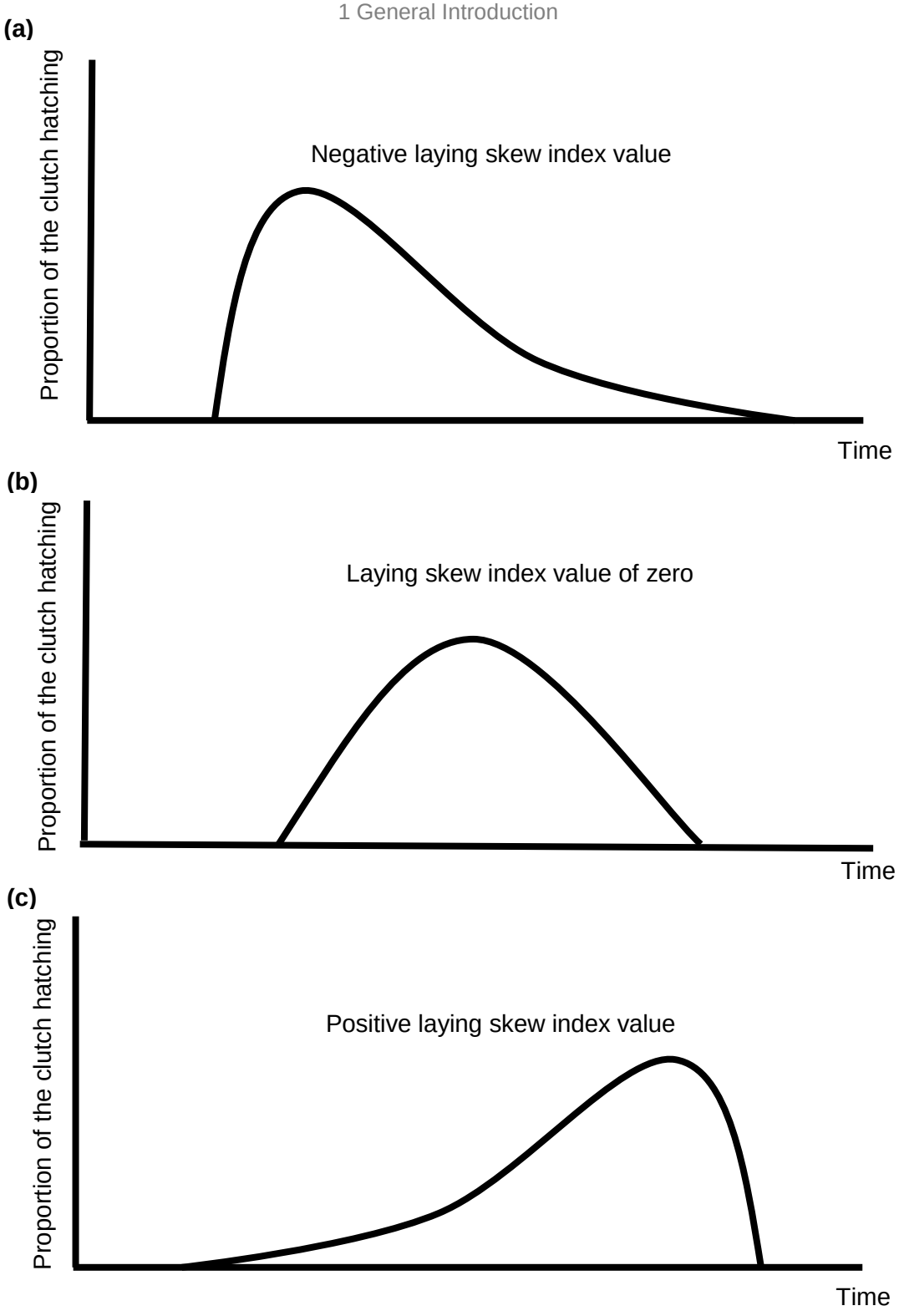



Figure 1.2: The proportion of the total clutch hatching in each time interval against time for clutches with (a) a negative laying skew index value, (b) a value of zero and (c) a positive laying skew index value.

I used ImageJ to measure eggs (Abramoff *et al*., 2004). I only measured eggs that were lying flat against the bottom of the box. If there were several suitable eggs on a single scan, I randomly chose which ones to measure. I measured eggs from the second image on which they were present as they appear to shrink during the first hour after being laid. Each egg was measured three times and the mean length and width for each egg was used to calculate a prolate spheroid volume (V) using the equation $V = (1/6)\pi w^2 L$, where w is the width and L the length of the egg (Berrigan 1991).

In Chapter 5 I used a different approach to quantify laying patterns. I obtained scans in the manner detailed above. As before, I and counted the number of eggs on each scan then subtracted the number of eggs on the previous scan to determine the number of new eggs laid each hour. I then assigned a value $t_{ij}$ to each egg *ij* in clutch *i* representing the number of hours after the female encountered the carcass until that egg was laid ($d_1$). I subtracted the mean time of laying for each clutch, $t_i$, from the value for each egg, and calculated the second-order deviations ($d_2$) and third-order deviations ($d_3$) from the clutch-mean, $(t_{ij}-t_i)^2$, and $(t_{ij}-t_i)^3$, respectively. Clutch-specific averages of these measures are the within-clutch means, variances, and skews, respectively, for timing of egg laying. This method obtains information on laying times from each egg rather than just the first and last egg of the clutch when calculating laying spread, therefore each egg contributes to the statistical sample size rather than one value of spread and skew for each clutch. In this chapter, rather than addressing hypotheses regarding the degree of hatching asynchrony, where laying spread and skew index are adequate measures, I instead investigated maternal effects on higher moments of within-clutch distributions of laying times. Using these laying metrics derived from the timing of laying of each egg allows questions regarding selection on within-clutch variability to be addressed.

I measured eggs rather than measuring or weighing larvae at hatching because this would require days of continuous presence in the lab to remove each larva and measure it when it hatched. This would be unfeasible and would potentially damage the larvae. Eggs can be measured precisely from the scanned images and this is not invasive. Prior work shows that there is a strong positive correlation between larval weight at hatching and egg size ($n$ = 63, $r$ = 0.7, $P$ = 0.001) (Steiger, 2013). I counted and weighed the brood at dispersal and calculated average larval mass because this correlates strongly with adult body size (Lock *et al.*, 2004), which is ecologically relevant because it influences competitive ability

(Otronen, 1988; Scott and Traniello, 1990; Trumbo, 1991; Robertson, 1993). I used pronotum width as a measure of adult size because it remains constant while mass fluctuates, it is widely used so it allows comparison with other studies, and it appears to be used by the beetles themselves to assess size when they interact with each other rather than mass (Safryn and Scott, 2000).

# Chapter 2

## Sexual conflict over parental care

This chapter has been published as referenced below, and this publication appears as Appendix C in this thesis:

Ford, L.E. & Smiseth, P.T. 2016. Asynchronous hatching provides females with a means for increasing male care but incurs a cost by reducing offspring fitness. *J. Evol. Biol*. **29**: 428-437.

**ABSTRACT**

In species with biparental care, sexual conflict occurs because the benefit of care depends on the total amount of care provided by the two parents while the cost of care depends on each parent's own contribution. Asynchronous hatching may play a role in mediating the resolution of this conflict over parental care. The sexual conflict hypothesis for the evolution of asynchronous hatching suggests that females adjust hatching patterns in order to increase male parental effort relative to female effort. I tested this hypothesis in the burying beetle *Nicrophorus vespilloides* by setting up experimental broods with three different hatching patterns: synchronous, asynchronous and highly asynchronous broods. As predicted, I found that males provided care for longer to asynchronous broods whereas the opposite was true of females. However, I did not find any benefit to females of reducing their duration of care in terms of increased lifespan or reduced mass loss during breeding. I found substantial negative effects of hatching asynchrony on offspring fitness as larval mass was lower and fewer larvae survived to dispersal in highly asynchronous broods compared to synchronous or asynchronous broods. My results suggest that, even though females can increase male parental effort by hatching their broods more asynchronously, females pay a substantial cost from doing so in terms of reducing offspring growth and survival. Thus, females should be under selection to produce a hatching pattern that provides the best possible trade-off between the benefits of increased male parental effort and the costs due to reduced offspring fitness.

## 2.1 INTRODUCTION

Sexual conflict, defined as a divergence in the evolutionary interests of individuals of the two sexes (Parker, 2006), is now recognised as ubiquitous in a variety of contexts, including mating and parental care (Arnqvist and Rowe, 2005). In species with biparental care, there is conflict over how much care each parent should provide because the benefit of care depends on the total amount of care provided by the two parents while the cost of care depends on each parent's own contribution (Lessells, 2012). As a consequence of this conflict, each parent is expected to minimise its costs of care by shifting as much of the workload as possible over to its partner (Trivers, 1972). One mechanism that might play a role in mediating the resolution of sexual conflict over parental care is asynchronous hatching, which occurs when the offspring from a single reproductive event hatch over an extended

period of time (Clark and Wilson, 1981). The sexual conflict hypothesis for the evolution of asynchronous hatching suggests that females adjust hatching patterns in order to increase male parental effort relative to female effort (Slagsvold and Lifjeld, 1989). Female birds can control hatching patterns by altering the timing of the onset of incubation: the brood hatches synchronously if the onset of incubation occurs after the clutch has been completed, while it hatches asynchronously if incubation begins before the last egg has been laid (Clark and Wilson, 1981). The initial version of this hypothesis proposed that the female benefits from hatching the young more asynchronously by making the male start feeding the young as soon as the earliest offspring hatch such that he continues feeding for longer than with a synchronous brood (Slagsvold and Lifjeld, 1989). However, a later version (sometimes termed the 'exploitation of mate hypothesis', Slagsvold *et al.,* 1995), suggested that the female benefits from hatching the young more synchronously because the male has to contribute more effort to prevent the brood from starving due to the peak in demand of each offspring occurring simultaneously.

The hypothesis that asynchronous hatching plays a role in mediating the resolution of sexual conflict over parental care predicts that the female should gain a fitness benefit from adjusting hatching patterns by increasing her partner's contribution towards parental care, thereby allowing her to reduce her own contribution. Previous studies on birds, which have tested this hypothesis by manipulating the degree of hatching asynchrony, have found mixed evidence. In support of the exploitation of mate hypothesis, Slagsvold (1997) found that males contributed more care towards synchronous broods while females reduced their contribution relative to asynchronous broods. Other studies have been unable to detect a consistent difference in male feeding rate between synchronous and asynchronous broods (Hillström, 1992; Amundsen, 1993; Hébert and Sealy, 1993; Stoleson and Beissinger, 1997). Furthermore, Slagsvold *et al*. (1994) found that synchronous hatching increased female survival in the subsequent year while asynchronous hatching increased male survival. In contrast, Stoleson and Beissinger (1997) found no difference in survival of male and female parents raising synchronous or asynchronous broods. Asynchronous hatching and biparental care are not unique to birds, but also occur in some insects (Nalepa, 1988; Müller and Eggert, 1990) and reptiles (While *et al.*, 2007). Thus, to improve our understanding of how asynchronous hatching contributes towards the resolution of sexual conflict over parental care, there is now a need to extend this work to nonavian systems.

Burying beetles of the genus *Nicrophorus* exhibit hatching asynchrony similar to that of many altricial birds (Müller and Eggert, 1990; Smiseth *et al.*, 2006; Takata *et al.*, 2015). These beetles breed on small vertebrate carcasses, which are buried underground (Scott, 1998b). Females lay eggs in the surrounding soil (Pukowski, 1933). In contrast to birds, burying beetles do not incubate the eggs. Instead, the asynchronous hatching pattern is determined by the period of time over which the eggs are laid, which is termed 'laying spread' (Smiseth *et al*., 2006; Takata *et al.*, 2015) and the extent to which laying is skewed towards the earlier part of the laying period, which is termed 'laying skew' (Smiseth *et al*., 2008) Thus, females can control the hatching pattern simply by adjusting laying spread and laying skew. In *Nicrophorus vespilloides* (Herbst), the mean interval between the hatching of the first and last larvae of a brood (i.e., hatching spread) is 30 hours. Given that the larvae disperse into the soil around 6 days after hatching, the hatching spread is considerable relative to the amount of time the larvae spend on the carcass (Smiseth *et al*., 2006). *Nicrophorus vespilloides* exhibits facultative biparental care (Bartlett, 1988). Either parent is capable of raising the brood alone, providing the opportunity for one parent to desert the brood and leave the other to care for the offspring (Bartlett, 1988). Parents provide care by preparing the carcass, defending it and the brood from predators and conspecifics, applying antimicrobials to the carcass, and provisioning the larvae with pre-digested carrion (Eggert *et al*., 1998; Rozen *et al.*, 2008; Walling *et al*., 2008; Arce *et al*., 2012). Sexual conflict over parental care occurs if parents benefit from reducing their investment in the current brood by increasing their survival and future reproductive success or by increasing the chances of finding another mate during the breeding season (Maynard Smith, 1977). *Nicrophorus vespilloides* appears to fulfil these criteria because there is a cost associated with providing care (Ward *et al*., 2009) and both sexes can breed more than once in a season (Bartlett and Ashworth, 1988) without any delay after rearing a brood (Scott and Traniello, 1990).

I conducted two experiments to test the sexual conflict hypothesis in *N. vespilloides*. Previous work on the resolution of sexual conflict over parental care highlights the distinction between evolutionary and facultative responses when studying how a focal parent adjusts its care to a change in the partner's workload, termed 'sealed-bids' and 'negotiation', respectively (Lessells, 2012). Thus, in Experiment 1, I tested whether females facultatively adjust hatching patterns in order to manipulate males to increase their contribution to parental care. Given that

biparental care in *N. vespilloides* is facultative, females may adjust hatching patterns depending on whether the male partner is present or absent at the start of breeding. The presence or absence of the male might provide females with a reliable cue as to whether a male is likely to assist in providing care for the larvae once the eggs have hatched. I recorded the timing of oviposition of females laying in the presence or absence of a male using scanners to minimise interference while females lay eggs. If females facultatively adjusted hatching patterns, I predicted that laying spread would differ when the male was present compared to when he was removed. In Experiment 2, I tested whether variation in the hatching pattern influences the male's contribution towards parental care and whether there is a benefit to females should the male make a greater contribution to parental care. Burying beetles do not differentiate between their larvae and larvae produced by other females as long as the larvae are introduced after their own eggs have hatched (Müller and Eggert, 1990). This allows me to use a cross-fostering design where I provided females with foster broods of a standardised brood size and a particular degree of asynchrony. I set up broods with three different hatching patterns (synchronous, asynchronous and highly asynchronous broods) and recorded how long each parent remained with the brood as a proxy for the amount of parental care. I assessed the fitness consequences for the parents by measuring effects on the survival and growth of the larvae and on the longevity and mass change of the parents. If asynchronous hatching plays a role in mediating the resolution of sexual conflict over parental care, I predicted that females would reduce their duration of care in broods with a greater hatching spread, with a corresponding increase in male care. I expected that reducing the amount of effort they invest in parental care would lead to a fitness benefit for females, such as an increase in the female's lifespan or a reduction in her loss of body mass during breeding.

## 2.2 METHODS

### 2.2.1 Study animals

The beetles used in this study were from an outbred laboratory population maintained at the University of Edinburgh. Beetles were housed individually in clear plastic boxes (124mm x 82mm x 22mm or 110mm x 110mm x 33mm). They were kept at 20 ± 2°C under constant lighting and were fed small pieces of organic beef twice a week. The beetles were aged 18-27 days post-eclosion at the start of the experiments.

### 2.2.2 Experimental procedures

2.2.2.1 *Experiment 1*

To determine whether females facultatively adjust laying patterns to increase the male's contributions towards care, I allowed females to lay eggs either in the presence or the absence of a male. I paired unrelated virgin males and females and placed them in a clear breeding box (17cm x 12cm x 6cm) containing 1cm of compost. I supplied each pair with a mouse carcass weighing 19.56-22.27g (previously frozen, supplied from Livefoods Direct Ltd, Sheffield, UK), which is within the range of vertebrate carcasses utilised by beetles in the wild (range: 1-37g; Müller *et al*., 1990b; Smiseth and Moore, 2002). I removed the male from half of the boxes after 6 hours, while leaving the male with the female in the remaining boxes (male present $n$ = 26, male absent $n$ = 24). Previous work suggests that parents respond to the absence of their partner within 45 minutes of removal (Steiger and Müller, 2010). Thus, given that the first eggs were laid after an average of 24 hours after pairing, females had ample time (on average 18 hours) to notice the male's absence before they began oviposition. Eggs are visible at the bottom of the breeding box and can be seen on images obtained by placing the boxes on flat-bed scanners (Canon Canoscan 9000F Mark II). In the small amount of soil used, the visible number of eggs is very similar to the actual clutch size (Monteith *et al*., 2012). I scanned the breeding boxes every hour using Vuescan professional edition software (Hamrick Software) until after the completion of oviposition. From the scanned images, I counted the number of new eggs laid each hour to determine the laying spread (the time between the first and last egg being laid) and the clutch size. I calculated a laying skew index (based on the hatching skew index of Smiseth *et al*., 2008) for each brood using the following formula: $\Sigma((t_i - t_m)/t_m)p_i$, where $p_i$ is the proportion of the total clutch laid each hour, $t_i$ is the time interval starting from the initiation of oviposition and $t_m$ is the middle of the laying period. To account for possible effects due to female and male body size, I also measured the pronotum widths of the parents using a Mitutoyo Absolute Digimatic calliper with a precision of 0.01mm. I set up 86 pairs initially but in analyses I excluded all pairs where either the eggs did not hatch ($n$ = 26) or there were technical problems with the scanner ($n$ = 10).

#### 2.2.2.2 *Experiment 2*

In order to investigate the influence of hatching patterns on the duration of care provided by males and females, I used a 2x3 fully factorial design with male presence versus absence and hatching spread (synchronous, asynchronous or highly asynchronous hatching) as the main factors. To set up the broods, I weighed virgin beetles, paired females with unrelated males, and placed each pair in a clear breeding box (17cm x 12cm x 6cm) containing 1cm of compost. I provided each pair with a mouse carcass weighing 19.37-22.22g (previously frozen, supplied from Livefoods Direct Ltd, Sheffield, UK). In half of the trials, I removed the male 6 hours after I provided the pair with a carcass, which is before the female had initiated egg laying. In the remaining trials, the male was left with the female during egg laying. In the interval between the end of egg laying and the start of hatching (i.e., 54-66h after pairing), I moved the remaining parents and the prepared carcass to a new box with fresh soil, while the eggs were left to develop in the original box. The larvae hatching from these eggs were then used to generate experimental foster broods. To ensure that I had an ample supply of foster larvae to generate the experimental broods, I set up additional donor pairs for breeding on the same day as the experimental pairs. I also set up some additional donor pairs over the subsequent two days. As soon as possible after their own larvae began to hatch, I provided breeding beetles with experimental foster broods that differed with respect to hatching spread (Smiseth and Morgan, 2009). The experimental broods were comprised of larvae that were unrelated to the foster parents and that were derived from up to four different donor females. The larvae were newly hatched and had not previously received any parental care form other individuals. Caring parents always received a total of 20 larvae, which is similar to the mean brood size of 21 larvae in this species (Smiseth and Moore, 2002). I weighed the larvae before placing them on the carcass as a measure of prenatal maternal investment. I generated synchronous broods by providing parents with 20 larvae at the same time. I generated asynchronous broods by providing parents with 10 larvae initially and then an additional 10 larvae 24 hours later. Finally, I generated highly asynchronous broods by providing parents with 10 initial larvae followed by 10 additional larvae 48 hours later. Thus, synchronous broods had a hatching spread of 0h, while asynchronous broods had a hatching spread of 24h and highly asynchronous broods had a hatching spread of 48h. This is within the natural variation of hatching spread, which can extend up to 56 hours in this species with a mean of around 30

hours (Smiseth *et al*., 2006). The total sample size in the experiment was $n$ = 126. The sample sizes for each treatment were as follows: $n$ = 20 for synchronous brood with male present, $n$ = 22 for synchronous brood with male absent, $n$ = 20 for asynchronous brood with male present, $n$ = 22 for asynchronous brood with male absent, $n$ = 22 for highly asynchronous brood with male present, and $n$ = 20 for highly asynchronous brood with male absent.

I used the amount of time that each parent spent with the brood from the arrival of the first larvae as a proxy for the amount of care they provided (Boncoraglio and Kilner, 2012; Smith *et al.*, 2014). I checked each box twice a day (at 09:00h and 17:15h) to determine whether the parent was present or absent from the brood chamber. If a parent was absent in two consecutive observations, I regarded it as having deserted the brood (Smith *et al*., 2014). Once the deserting parent had been removed from the box, I weighed it to record its post-breeding body mass. If parents did not desert the brood before larval dispersal (defined as when the majority of larvae left the carcass), I weighed and removed them at the time when the larvae dispersed from the carcass. I placed all parents in individual boxes upon removal from the breeding box, and fed them small pieces of organic beef twice a week. I recorded the number of larvae dispersing from each brood and weighed the entire brood to obtain the total dispersing brood mass, from which I calculated the average larval mass. I then placed the larvae in a box (17cm x 12cm x 6cm) filled with soil and allowed them to eclose. As keeping all offspring would amount to an excessive workload, I randomly selected one male and one female offspring from each brood upon eclosion and retained them to record potential effects on lifespan. I recorded the sex and pronotum width of the other offspring. I checked parents and retained offspring at least three times a week to obtain the approximate age of death, and measured their pronotum widths using a Mitutoyo Absolute Digimatic calliper.

### 2.2.3 Statistical analyses

I carried out the statistical analyses in R (R Core Team, 2014). I selected model families and link functions based on graphical model validation and AIC values where appropriate. I carried out model refinement through backwards stepwise deletion using the *drop1* function ($P$-values based on $F$ or $\chi$ statistics). To analyse the results of Experiment 1, I constructed generalised linear models to investigate the effect of male removal on laying spread (Gamma family, inverse link function)

and laying skew (Gaussian family, identity link function). I used Kendall's Tau correlation to test for a correlation between laying spread and laying skew. I constructed generalised linear models to investigate the influence of hatching pattern on parental care and aspects of parent and offspring fitness studied in Experiment 2. Table 2.1 shows the full models and the model family and link function used in each model. I used Kendall's Tau correlation to test for a correlation between male presence and female presence. I used Wilcoxon signed ranks test to determine whether female presence was affected by removal of the male. I also used Wilcoxon signed ranks tests to compare female age at death and male age at death between treatments where the male was removed or was allowed to remain with the brood. Finally, I compared total parental presence between hatching patterns using a Kruskal-Wallis test.

## 2.3 RESULTS

### 2.3.1 Experiment 1

In contrast to what I predicted if females facultatively adjusted their egg laying to the presence or absence of the male, the removal of the male before oviposition did not significantly affect average laying spread ($F_{1,48} = 0.09$, $P = 0.768$). Laying skew was also not significantly affected by male removal ($F_{1,48} = 2.60$, $P = 0.114$) and there was no correlation between laying spread and laying skew ($z = 0.30$, $P = 0.763$). Laying spread was greater for larger clutches ($F_{1,48} = 21.57$, $P < 0.0001$) and there was a non-significant trend towards a greater laying spread when oviposition commenced earlier ($F_{1,48} = 4.00$, $P = 0.0514$).

### 2.3.2 Experiment 2

Consistent with what I predicted, the hatching pattern had a significant effect on the amount of time that the male was caring for the brood (the number of observations the parent was present out of total number of observations when there were larvae on the carcass) ($t_{58} = 3.18$, $P = 0.0024$). The male remained for longest when caring for highly asynchronous broods (Figure 2.1a). In contrast, the female deserted highly asynchronous broods sooner ($F_{1,60} = 5.41$, $P = 0.0234$, Figure 2.1b). Although the amount of time the male was present was highly negatively correlated with the amount of time the female was present ($z = -3.27$, $P = 0.0011$), the amount of time the female was present was not significantly affected by the removal of the male before oviposition ($W = 2246$, $P = 0.171$). This suggests that the male responds by

delaying his desertion of the brood when the female deserts first, while the female's decision was not affected by male desertion. Furthermore, the male responded differently to female desertion depending on the hatching pattern (interaction between hatching pattern and amount of time females provided care: $F_{1,58} = 9.68$, $P = 0.0029$); the male responded more strongly to female desertion when he was caring for highly asynchronous broods. Despite this, the total amount of presence by male and female parents was similar across all three hatching patterns ($\chi^2 = 4.74$, $P = 0.0934$). There was no significant effect of female pronotum width ($F_{1,60} = 3.01$, $P = 0.0938$) or male pronotum width ($F_{1,58} = 0.00$, $P = 0.997$) on the duration of care provided.

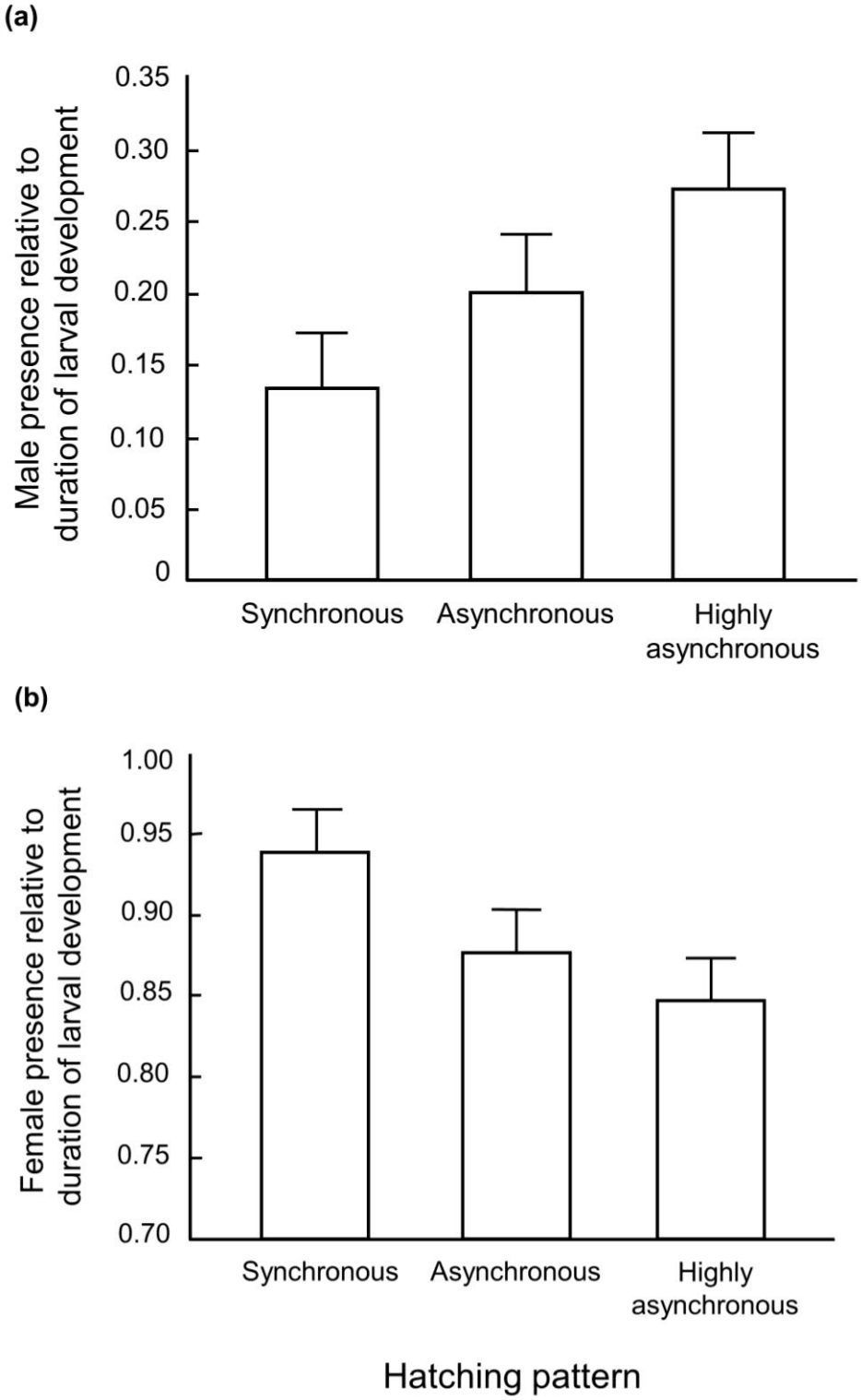



Figure 2.1: Mean duration of (a) male presence and (b) female presence with the brood as a proportion of the total time larvae were on the carcass for synchronous, asynchronous and highly asynchronous broods. Error bars represent standard errors.

In contrast to what I predicted, I found no evidence that a reduction in the duration of female care led to an increase in the female's own lifespan or a reduction in her loss in body mass during breeding. The female's adult lifespan (number of days from eclosion to death) was not significantly affected by the hatching pattern ($F_{1,123} = 1.16$, $P = 0.283$). Furthermore, female lifespan was not significantly affected by the absolute duration of time the female spent with the brood ($F_{1,123} = 0.16$, $P = 0.695$). Similarly, male lifespan was not significantly affected by the hatching pattern ($F_{1,60} = 0.38$, $P = 0.541$) or the amount of time spent with the brood ($F_{1,60} = 0.06$, $P = 0.814$). Females had a significantly longer lifespan than the males ($W = 4492$, $P < 0.0001$). Female lifespan was not significantly affected by whether the male was present or absent ($W = 1934$, $P = 0.931$), the males survived slightly longer when allowed to remain with the brood ($W = 1519$, $P = 0.0322$). I found that most parents (95% of all parents) gained mass during the breeding attempt. Mass change was not significantly affected by hatching pattern ($F_{1,185} = 0.41$, $P = 0.521$), sex ($F_{1,185} = 0.18$, $P = 0.6697$), or the interaction between sex and hatching pattern ($F_{1,185} = 0.01$, $P = 0.907$). However, mass change was greater when parents remained with the brood for longer ($F_{1,185} = 6.44$, $P = 0.0120$) and was also greater for parents that initially had a lower body mass ($F_{1,185} = 11.15$, $P = 0.0010$).

Hatching patterns influenced larval survival as a greater number of larvae survived to dispersal in synchronous or asynchronous broods than in highly asynchronous broods ($\chi^2_{1,123} = 12.76$, $P = 0.0004$, Figure 2.2). Larval survival was not significantly affected by male removal ($\chi^2_{1,123} = 0.66$, $P = 0.418$). However, the number of surviving larvae was greater when the larvae had a greater initial mass at the time when they were introduced to the carcass ($\chi^2_{1,123} = 26.40$, $P < 0.0001$). The duration of larval development was also affected by hatching pattern ($F_{1,124} = 22.12$, $P < 0.0001$) as highly asynchronous broods took on average 0.63 days longer to reach dispersal than synchronous broods. Offspring lifespan was not significantly affected by hatching pattern ($F_{1,248} = 0.80$, $P = 0.371$), male removal ($F_{1,248} = 0.021$, $P = 0.886$), the total duration of parental care ($F_{1,248} = 0.29$, $P = 0.588$), the offspring's sex ($F_{1,248} = 3.28$, $P = 0.071$), or offspring pronotum width ($F_{1,246} = 0.33$, $P = 0.565$). The mean mass of a larva at dispersal decreased with increasing hatching spread ($F_{1,123} = 36.05$, $P < 0.0001$, Figure 2.3) and increased with increasing total duration of parental presence ($F_{1,123} = 14.63$, $P = 0.0002$). However, mean larval mass was not significantly affected by male removal ($F_{1,123} = 0.16$, $P = 0.694$). Similarly, the mean pronotum width of the offspring from each brood also decreased

with increasing hatching spread ($F_{1,124} = 22.50$, $P < 0.0001$) and was not significantly affected by male removal, although there was a non-significant trend towards greater offspring pronotum widths when the male was allowed to remain with the brood ($F_{1,124} = 3.91$, $P = 0.0502$). The standard deviation in offspring pronotum width increased with increasing hatching spread ($F_{1,124} = 79.10$, $P < 0.0001$).

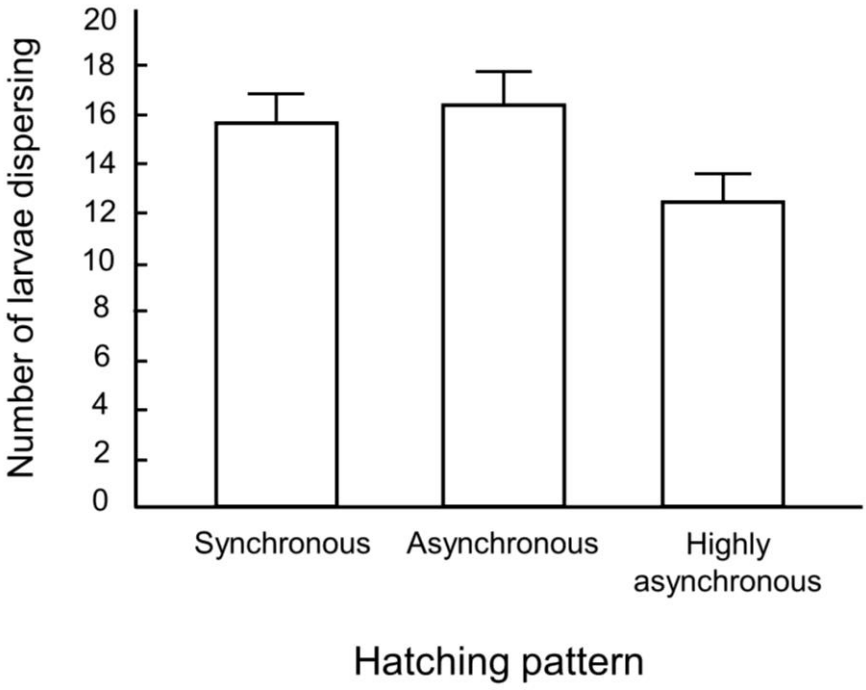



Figure 2.2: Mean number of larvae dispersing from the carcass for synchronous, asynchronous and highly asynchronous broods. Error bars represent standard errors.

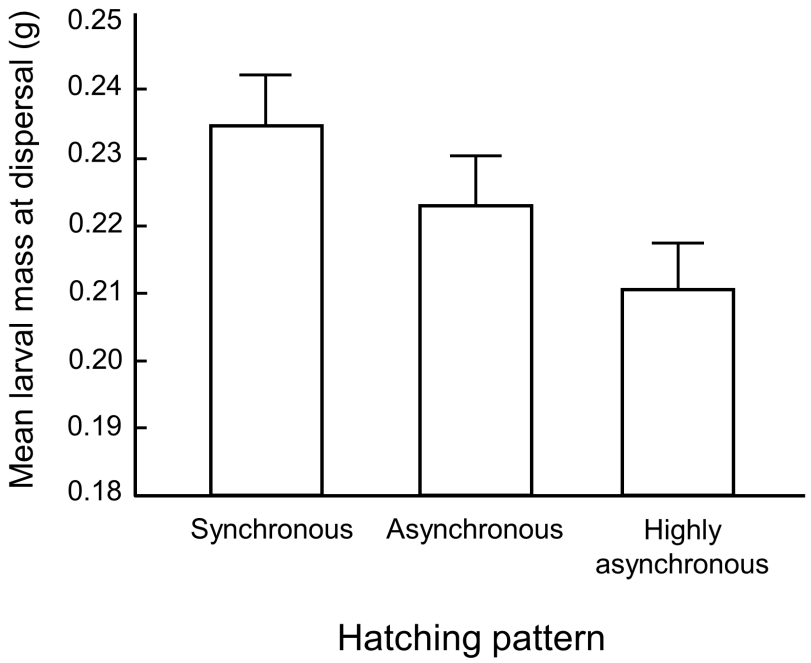



Figure 2.3: Mean mass of a larva at dispersal (total brood mass divided by number of larvae) for synchronous, asynchronous and highly asynchronous broods. Error bars represent standard errors.

Table 2.1: Summary of generalised linear models showing all terms included in the full model, and model families and link functions. Age refers to the age of an individual at the time of breeding.

| Response variable | Full model | Family | Link function |
|---|---|---|---|
| Female time present | Hatching pattern + Carcass mass + Female body size + Female age | Quasi | 1/mu^2 |
| Male time present | Female time present*Hatching pattern + Carcass mass + Male body size + Male age | Quasi | Identity |
| Parent proportional mass change | Sex*Hatching pattern +Time present + Carcass mass + Initial mass | Gaussian | Identity |
| Female lifespan | Hatching pattern + Female time present + Female body size + Female age | Gamma | Identity |
| Male lifespan | Hatching pattern + Male time present + Male body size + Male age | Gamma | Identity |
| Number of larvae dispersing | Hatching pattern + Male presence/absence + Total parental presence + Carcass mass + Average initial larval mass + Duration of larval development | oisson | Identity |
| Mean larval mass at dispersal | Hatching pattern*Male presence/absence + Total parental presence + Carcass mass + Average initial larval mass | Inverse gaussian | Identity |
| Mean offspring pronotum width | Hatching pattern + Male presence/absence + Carcass mass | Gaussian | Inverse |
| SD in offspring pronotum width | Hatching pattern + Male presence/absence + Carcass mass | Inverse gaussian | Log |
| Duration of larval development | Hatching pattern + Male presence/absence | Gaussian | Identity |
| Offspring age at death | Hatching pattern + Male presence/absence + Offspring body size + Sex + Total parental presence | Gamma | Inverse |

## 2.4 DISCUSSION

In Experiment 1, I found that laying spread and laying skew were similar regardless of whether the male was experimentally removed or allowed to remain with the female during oviposition. This finding suggests that females do not adjust hatching patterns facultatively in response to male removal in *N. vespilloides*, and thus that the degree of asynchronous hatching represents an evolutionary response to sexual conflict over parental care. I am unaware of any evidence from the literature showing that females adjust hatching patterns facultatively in response to the absence or removal of the male. The absence of evidence for facultative responses in *N. vespilloides* and birds might reflect that there has not been strong selection on females to adjust hatching patterns depending on whether the male is present or absent, possibly reflecting that females normally are assisted by a male partner (Scott, 1998b; Cockburn, 2006). If the hatching pattern is an evolutionary response to sexual conflict over parental care, I might expect the optimal hatching pattern for the female to depend on male parental effort and the optimal parental effort for the male to depend on the hatching pattern. The outcome of this co-evolutionary process might be for females to evolve a hatching pattern that is associated with an evolutionary increase in male parental effort to relative to female parental effort.

In Experiment 2, I found that males remained for longer when caring for highly asynchronous broods than when caring for asynchronous and synchronous broods, while females in contrast deserted earlier when caring for highly asynchronous broods. This difference between males and females is consistent with the prediction of the sexual conflict hypothesis (Slagsvold and Lifjeld, 1989), and suggests that females could increase male contributions to parental care by laying the eggs more asynchronously. I suggest two possible mechanisms for the observed effect of hatching pattern on the duration of paternal care. Firstly, males may prolong their involvement in care when caring for highly asynchronous broods in response to the female deserting earlier. In support of this suggestion, I found that males remained with the brood for longer when their partner deserted earlier, and similar results showing that males adjust their contribution to the absence of the female have been found in previous studies on the amount of care (Fetherston *et al*., 1994; Smiseth and Moore, 2004; Rauter and Moore, 2004; Smiseth *et al*., 2005; Suzuki and Nagano, 2009; Smith *et al*., 2014). I found that males responded more strongly to female desertion in highly asynchronous broods, but that the total

duration of parental care was similar across all hatching patterns. Males may respond by staying for longer should the female desert early on in order to prevent conspecifics from usurping the carcass and killing the brood (Trumbo, 1990a). Secondly, my results may reflect that males reduce their effort later if they contributed more towards care early on (Amundsen, 1999). Smiseth and Morgan (2009) found that the peak in brood demand is significantly higher in synchronous broods than in highly asynchronous broods. Thus, males may initially contribute more towards parental care when caring for synchronous broods while there is a high demand, and may desert the brood earlier as a consequence of their higher initial contribution. If so, males might potentially be contributing a similar amount of care towards synchronous and asynchronous broods by remaining for longer but providing lower levels of care in the latter broods. I have no information on the amount of care given that I used residency time as a proxy for parental effort instead of behavioural observations. Thus, further work is now needed to examine the mechanisms whereby hatching patterns influence the duration of paternal care.

I found that the duration of female care was not influenced by the removal of the male before larvae were present on the carcass, suggesting that females do not adjust their timing of desertion in response to male behaviour. Similar results showing that females do not respond to male removal have been found in previous studies on *N. vespilloides* (Smiseth *et al.*, 2005) and the closely related *N. orbicollis* (Rauter and Moore, 2004). I also found that females deserted highly asynchronous broods earlier than asynchronous or synchronous broods. This finding might reflect that females value highly asynchronous broods less highly given that I also found that these broods produce fewer and smaller surviving larvae. Previous work shows that females adjust their provisioning behaviour in response to changes in the demand of the older larvae in the brood rather than the entire brood (Smiseth and Morgan, 2009). Thus, females may be more sensitive to the requirements of older larvae, in which case they might desert the brood depending on the age of the older larvae in the brood rather the average age of the brood. The greater duration of female care seen in synchronous broods with 20 older larvae than in asynchronous and highly asynchronous broods with 10 older larvae is consistent with this suggestion. It is not known whether males are more responsive to the needs of older larvae than younger larvae, but given that they provide less care overall than females, they may not be under strong selection to discriminate between different-aged larvae. Further work is needed to examine whether there is a difference in how

males and females respond to the demand of the older larvae relative to the entire brood.

My study shows that asynchronous hatching had detrimental effects on the offspring's fitness. Firstly, I found that larval survival was lowest in highly asynchronous broods, as previously reported by Smiseth and Morgan (2009). This finding shows that high levels of hatching asynchrony increase the offspring's mortality. Secondly, I found that mean larval mass at dispersal and offspring pronotum width at eclosion were lower in highly asynchronous broods, which contrasts with previous studies reporting no effect of hatching patterns on larval mass at dispersal (Smiseth *et al*., 2008; Smiseth and Morgan, 2009). Such reductions in offspring size should have detrimental fitness consequences given that smaller offspring develop into smaller adults (Lock *et al.*, 2004), and that smaller adults are less likely to be successful in competition for breeding resources (Otronen, 1988; Scott and Traniello, 1990; Trumbo, 1991; Robertson, 1993). Thirdly, I found that the duration of larval development was longer for highly asynchronous broods, potentially increasing the vulnerability of the brood to predation or infanticidal intruders. The detrimental effects of asynchronous hatching on offspring survival and growth are likely to be the outcome of asymmetric sibling competition caused by asynchronous hatching. Smiseth *et al*. (2007a) found that older larvae in asynchronous broods grow better than younger larvae as long as the parents provide care, suggesting that parental care somehow exacerbates asymmetric sibling competition. Previous work on burying beetles suggests that older larvae consistently have higher survival and greater body mass than younger larvae regardless of hatching spread or skew (Takata *et al*., 2014), reflecting that they receive more parental care (Smiseth *et al*., 2007a; Smiseth and Moore 2008; Takata *et al*., 2013; Andrews and Smiseth, 2013). Similar detrimental effects of asymmetric sibling competition on offspring survival and growth have also been reported in birds (Clark and Wilson, 1981). These detrimental fitness consequences of asynchronous hatching have important consequences for the sexual conflict hypothesis because, although hatching asynchrony provides females with a means for increasing the male's contribution to parental care, females can only do so by also reducing their offspring's size and survival. Thus, females should be under selection to produce an intermediate hatching pattern that provides the best possible trade-off between the benefits of increased male parental effort and the costs due to reduced offspring fitness.

I found that mean larval mass was greater when parents provided care for longer. Previous work has found that parental care improves offspring growth, particularly during the early stages of larval development (Eggert *et al.*, 1998; Smiseth *et al*., 2003). Thus, caring for the brood for longer has positive effects on the offspring's fitness. The fact that one of the parents often deserts the brood prematurely therefore suggests that there must be some cost of providing care, such as reduced lifespan or increased loss of body mass, or that there are some benefits of deserting the brood, such as increased opportunities for breeding (Royle *et al.*, 2012). If the sexual conflict hypothesis is to be supported, the benefits that the female gains from increasing the male's contribution towards care for highly asynchronous broods, thereby allowing the female to reduce her own costs of care, should outweigh the detrimental effect of reduced offspring fitness. Currently, it is unclear what costs parents incur from providing care in burying beetles. There does not appear to be an immediate physiological cost of breeding given that almost all parents in my study gained mass during breeding – a result that also has been found in the closely related *N. orbicollis* (Scott and Traniello, 1990) – and that parents that remained with the brood for longer gained proportionally more mass. Furthermore, I did not find any long-term cost of caring, as there were no significant effects of the hatching pattern or the duration of parental care on female lifespan. In contrast to what was found by Boncoraglio and Kilner (2012), I found no significant effect of male presence after hatching on female lifespan. This may reflect that the benefit to females of being assisted by a male depends on the ecological context. For example, male presence could be detrimental if there is competition for food between parents and offspring on small carcasses because dispersing brood mass is lower for broods reared by males that gain mass (Scott and Gladstein, 1993) and females sometimes even kill males on very small carcasses (Bartlett, 1988). The carcasses used in the present study were large enough to support 20 larvae and thus there were probably sufficient resources for two parents to feed without depriving the offspring of food.

Although my study did not identify a benefit to the female of reducing her duration of care, it is possible that I was unable to detect such a benefit in my laboratory experiment. For example, females may benefit from deserting earlier if this reduces their risk of infection by microorganisms present on the carcass. I always used fresh carcasses in my experiment, but females in the field may breed on carcasses that have begun to decompose before the start of the breeding

attempt (Steiger *et al*., 2011). Furthermore, females may benefit from deserting earlier by reducing the risk of injury or death during fights with conspecifics. I always excluded competitors in my experiments, while females in the field may face both interspecific and intraspecific competitors, which may cause injury or death (Trumbo, 1990b). Thus, future work on the potential benefits to females from increasing the amount of male care should consider designs that mimic the harsher conditions these beetles face in the wild.

My study is the first to test the sexual conflict hypothesis in a nonavian species. I found some support for the sexual conflict hypothesis in the burying beetle *N. vespilloides*. As predicted, I found that males and females responded differently to hatching patterns: males provided care for longer in highly asynchronous broods whereas the opposite was true of females. My findings suggest that asynchronous hatching may play a role in the resolution of sexual conflict over parental care in *N. vespilloides*. However, I did not find any evidence that females benefitted from reducing their duration of care, and I found costs of high levels of hatching asynchrony in terms of reduced larval growth and survival. I argue that hatching asynchrony would only be a viable strategy for females to increase the male's contribution to care if the benefits to females from reducing their own costs of care outweigh the costs of reduced offspring fitness. I recommend that future studies on the sexual conflict hypothesis recognise the importance of assessing fitness consequences for parents and offspring in addition to studying changes in each parent's contribution towards parental care.

# Chapter 3

## The hurry-up hypothesis

This chapter has been published as referenced below, and this publication appears as Appendix D in this thesis:

Ford, L.E. & Smiseth, P.T. 2017. Asynchronous hatching in a nonavian species: a test of the hurry-up hypothesis. *Behav. Ecol*. **28**: 899-907.

**ABSTRACT**

The hurry-up hypothesis suggests that completing reproduction as soon as possible is favoured when the quantity or quality of resources used for breeding declines over time. However, completing reproduction sooner may incur a cost if it leads to an asynchronous hatching pattern that reduces overall growth and survival of offspring. Here, I present the first test of the hurry-up hypothesis in a nonavian system, the burying beetle *Nicrophorus vespilloides*, which breeds on small vertebrate carcasses. To this end, I conducted two experiments in which I provided females with an incentive to complete reproduction sooner by giving them carcasses that varied either with respect to decomposition (resource quality) or size (resource quantity). I recorded the delay until laying and measures of the laying pattern and fitness consequences for the offspring. As predicted, I found that larvae dispersed from the carcass earlier when females commenced oviposition sooner and that laying spread was greater when females commenced egg laying earlier. However, I found no evidence that females commenced egg laying earlier on either decomposed or larger carcasses. My results suggest that, although asynchronous hatching might emerge as a by-product of parents attempting to complete reproduction sooner, there is no evidence that females attempt to complete reproduction sooner under conditions where this would be favourable. My results are therefore inconsistent with the hurry-up hypothesis.

## 3.1 INTRODUCTION

In species where parents provide food or other forms of care for their dependent offspring, parents may be under selection to respond to deteriorating environmental conditions by shortening the time from the onset of breeding until the offspring reach independence (Hussell, 1972; Clark and Wilson, 1981; Slagsvold, 1986). This condition might be met when there is a decline in the quantity or quality of food resources (Hussell, 1972; Clark and Wilson, 1981; Slagsvold, 1986), an increase in predation rate on dependent offspring, or when climatic conditions worsen over time (Magrath, 1990). The hurry-up hypothesis was proposed to explain how parent birds might respond to deteriorating environmental conditions (Hussell, 1972; Clark and Wilson, 1981) but the hypothesis may apply in general across animal taxa. Female birds are constrained from laying more than one egg each day, which means that it takes them several days to complete a clutch. However, because avian eggs only start developing once parents start incubating, parents control embryonic

development by simply adjusting the onset of incubation (Clark and Wilson, 1981). Thus, if parent birds perceive that the peak in food supply will occur earlier than anticipated at the start of laying, they can accelerate offspring development by commencing incubation earlier instead of waiting until the clutch has been completed. However, studies on birds show that parental attempts to shorten the time until offspring independence in response to dwindling food supplies towards the end of the breeding season are also associated with greater levels of asynchronous hatching (Gibb, 1950; Van Balen, 1973; Nisbet and Cohen, 1975; Slagsvold, 1982, 1986; Hébert and McNeil, 1999).

The hurry-up hypothesis suggests that asynchronous hatching emerges as a nonadaptive by-product due to parent birds being under selection to commence incubation before the clutch has been completed (Clark and Wilson, 1981). There is good evidence that hatching asynchrony can incur substantial fitness costs because it often leads to asymmetric sibling competition with a detrimental impact on the survival and/or growth of the last offspring to hatch (Lack, 1947; Clark and Wilson, 1981; Hillström and Olsson, 1994; Smiseth *et al*., 2007a). Thus, parents should be under selection to balance the benefits of shortening the time until the first offspring reach independence against the costs of producing an asynchronous hatching pattern that reduces the overall growth and survival of offspring. Furthermore, parents may be under selection to offset any undesirable fitness consequences of asymmetric sibling competition (Clark and Wilson, 1981). For example, in several birds with asynchronous hatching, parents produce larger eggs toward the end of the laying sequence (Schrantz, 1943; Kendeigh *et al*., 1956; Holcomb, 1969; Howe, 1976, 1978; Bryant, 1978). This may compensate for some of the fitness costs of asymmetric sibling competition given that offspring hatching mass is positively correlated with egg size (Krist, 2011) and chicks hatching from heavier eggs have higher early growth (Hillström, 1999).

Although the hurry-up hypothesis was proposed for birds where parents incubate their eggs, it may also apply to nonavian species where eggs develop without incubation, provided that there is a trade-off between commencing oviposition soon after the initiation of reproduction and laying eggs synchronously. Burying beetles of the genus *Nicrophorus* are an ideal nonavian study system in which to test the hurry-up hypothesis because they exhibit hatching asynchrony similar to that of many altricial birds (Müller and Eggert, 1990; Smiseth *et al*., 2006; Takata *et al*., 2015). These beetles breed on carcasses of small vertebrates,

which are buried underground (Scott, 1998b). Females lay eggs in the surrounding soil (Pukowski, 1933) and the hatching pattern is determined by the period of time over which the eggs are laid, termed “laying spread” (Smiseth *et al*., 2006; Takata *et al*., 2015). The degree of hatching asynchrony is highly variable in *N. vespilloides*, ranging from 16 to 56h with a mean of 30h, which is considerable relative to the duration of embryonic development (59h) and the duration of parental food provisioning (72h) (Smiseth *et al*., 2006). Burying beetles could potentially reduce the delay until the offspring reach independence by starting to lay eggs sooner after encountering the carcass. However, because females do not mature their oocytes until they secure a carcass for breeding (Wilson and Knollenberg, 1984), starting to lay very soon after encountering the carcass may be costly as females may not have consumed enough resources to mature all of their oocytes before commencing oviposition. If this is the case, starting to lay soon after encountering the carcass may be associated with a greater laying spread and a greater level of hatching asynchrony if there is a trade-off between accelerating the maturation of some oocytes and maturing all oocytes synchronously. This may be undesirable due to increased mortality of the last hatched offspring in asynchronous broods (Smiseth *et al*., 2008; Takata *et al*., 2014; Ford and Smiseth, 2016).

Here, I present the results of two experiments designed to test the hurry-up hypothesis in *N. vespilloides*. I provided female beetles with an incentive to shorten the time until offspring independence in response to variation in either resource quality or resource quantity. I used carcass decomposition as a proxy for resource quality while I used carcass size as a proxy for resource quantity. I predicted that females would shorten the time until offspring independence when breeding on decomposed carcasses because the value of the carcass should decrease over time due to an increase in microbial load. Indeed, carcass decomposition has a detrimental effect on larval growth and survival (Rozen *et al*., 2008) and egg survival (Jacobs *et al*., 2014). Meanwhile, I predicted that females would start to lay sooner when breeding on larger carcasses because larger carcasses are more difficult to roll into a ball; so, it is more difficult to control microbial activity due to the larger surface area remaining exposed (Trumbo, 1992). Furthermore, it is more difficult for the parents to keep the carcass coated with antimicrobial secretions, which may provide an incentive to complete reproduction sooner on larger carcasses, before decomposition of the carcass becomes detrimental to the larvae. In addition, larger carcasses are more likely to be usurped by other burying beetles (Trumbo, 1991),

which will kill the entire resident brood (Trumbo, 1990a). Completing reproduction as soon as possible is particularly important on large carcasses because the risk of usurpation is not constant; in *N. orbicollis* the risk of takeovers decreases after 5 days (Robertson, 1993), possibly because depletion of the carcass due to larvae feeding makes it more difficult for free-flying beetles to detect. Moreover, the brood is less likely to be killed by a vertebrate scavenging the carcass if reproduction is completed sooner (Trumbo, 1992). Here, I addressed the following 4 questions: 1) Are the assumptions of the hurry-up hypothesis met in this system? If so, females could shorten the time to offspring independence by commencing oviposition sooner and there would be a trade-off between commencing oviposition sooner and laying the eggs more synchronously. 2) Do females adjust egg laying in response to variation in environmental conditions? I predicted that females breeding on decomposed or larger carcasses would shorten the time until offspring independence by commencing oviposition sooner and that they would lay their eggs more asynchronously. 3) Is breeding success influenced by females commencing oviposition sooner and by patterns of egg laying? I predicted that starting to lay sooner would be beneficial and that asynchrony would be detrimental in terms of the size, number or survival of larvae. 4) Do females compensate for the fitness costs of asymmetric sibling competition? If so, I predicted that females would lay larger eggs towards the end of the laying sequence.

## 3.2 METHODS

### 3.2.1 Study animals

The beetles used in this study were from an outbred laboratory population maintained at the University of Edinburgh. Beetles were housed individually in clear plastic boxes (124mm × 82mm × 22mm). They were kept at 20 ± 2°C under constant light and were fed small pieces of organic beef twice a week. The beetles were sexually mature and of prime reproductive age (18-26 days post-eclosion) at the start of the experiments.

### 3.2.2 Experimental procedures

The aim of the carcass decomposition experiment was to determine whether females attempt to shorten the time until offspring independence when the carcass is at a more advanced stage of decomposition at the start of the breeding attempt and whether this is associated with greater levels of hatching asynchrony. I provided

females with either a fresh mouse carcass that had been thawed immediately before it was provided to the beetles (*n* = 36) or a decomposed carcass that had been left in the breeding box for 10 days before it was provided to the beetles (*n* = 35). The carcasses used in this experiment weighed 23.32–29.50g (mean 25.57g) when initially thawed. I initially set up pairs on 42 fresh and 59 decomposed carcasses but excluded all pairs where the eggs did not hatch (fresh carcasses: *n* = 3; decomposed carcasses: *n* = 21) and where there were technical problems (fresh carcasses: *n* = 3; decomposed carcasses: *n* = 3). The aim of the carcass size experiment was to determine whether females attempt to complete reproduction sooner on larger carcasses. I allowed beetles to breed on a range of carcasses (*n* = 82) from 4-27g, which is within the range of vertebrate carcasses utilized by beetles in the wild (range: 1-37g; Müller *et al*., 1990b). I initially set up 92 pairs but excluded 1 pair that failed to lay eggs and 9 pairs that failed to hatch larvae.

For both experiments, I paired unrelated virgin males and females and placed them in a clear breeding box (170mm × 120mm × 60mm) containing 1cm of moist compost. I supplied each pair with a previously frozen mouse carcass (supplied from Livefoods Direct Ltd, Sheffield, UK). Eggs are visible at the bottom of the breeding box and can be seen on images obtained by placing the boxes on flatbed scanners (Canon Canoscan 9000F Mark II, Canon Inc., Tokyo, Japan) (Ford and Smiseth, 2016). In the small amount of soil used, the visible number of eggs is very similar to the actual clutch size (Monteith *et al*., 2012). I scanned the breeding boxes every hour using Vuescan professional edition software (Hamrick Software, Sunny Isles Beach, FL) until the eggs hatched. In accordance with previous studies, I defined a clutch as comprised of all eggs laid before the first larva hatched (Müller, 1987; Steiger, 2013). From the scanned images, I counted the number of new eggs laid each hour to determine the laying spread (the time between the first and last egg being laid) and the clutch size (the number of eggs laid before the first egg hatched, Müller *et al*., 1990b). I excluded clutches where all eggs failed to hatch because females will continue to lay eggs if larvae do not arrive at the carcass, resulting in aberrant laying patterns (Müller, 1987). I also calculated a laying skew index reflecting the extent to which laying is skewed toward the earlier part of the laying period, using the formula $\Sigma((t_i–t_m)/t_m)p_i$, where $p_i$ is the proportion of the total clutch laid each hour, $t_i$ is the time interval starting from the initiation of oviposition and $t_m$ is the middle of the laying period (Smiseth *et al*., 2008). I recorded the number of days since pairing until the larvae dispersed from the carcass and

counted the number of larvae dispersing from each brood. At the time of dispersal, I also weighed the entire brood mass and calculated the average larval mass by dividing the brood mass by the number of larvae in the brood.

I measured egg size in the carcass decomposition experiment to determine whether females compensated for the consequences of asymmetric sibling competition caused by asynchronous hatching by increasing egg size across the laying sequence. Using ImageJ (Abramoff *et al*., 2004), I split the clutch approximately into thirds and measured the length and width of 3 eggs from the first third (first eggs), 3 eggs from the second third (middle eggs), and 3 eggs from the last third of the clutch (last eggs). I only measured eggs that were lying flat against the bottom of the box. If there were several suitable eggs on a single scan, I randomly chose which ones to measure. Three broods were excluded for the middle eggs because there were no suitable eggs to measure that had not already been measured as either first or last eggs due to small clutch sizes. Eggs were measured from the second image on which they were present as they appear to shrink during the first hour after being laid. Each egg was measured 3 times and the mean length and width for each egg was used to calculate a prolate spheroid volume (V) using the equation $V = (1/6)\pi w^2 L$, where w is the width and L the length of the egg (Berrigan 1991). I did not measure eggs in the carcass size experiment because laying asynchrony did not differ with carcass size once the greater clutch sizes on larger carcasses were taken into account and therefore I did not expect that there would be sufficient asymmetric sibling competition to elicit a response from females attempting to compensate.

### 3.2.3 Statistical analyses

I carried out the statistical analyses in R (R Core Team, 2014). I constructed generalized linear models. Graphical model validation showed that the residuals of the model were normally distributed and homoscedastic, that there was no over dispersion and that the model was not biased by unduly influential observations. Variance inflation factors confirmed that there was not excessive collinearity of the variables. I carried out model refinement through backwards stepwise deletion to determine the significance of each term comparing otherwise identical models either including or excluding the term of interest using likelihood ratio tests. I then removed the least significant term and repeated the process until only significant terms remained. I used correlation tests (Pearson's correlation test, or Kendall's Tau

correlation where the data did not fit a normal distribution) to investigate whether a shorter delay until laying after females encountered the carcass was associated with earlier larval dispersal or greater laying asynchrony.

I initially produced a model for each response variable containing only treatment to determine whether there was an effect of carcass decomposition or carcass size. I then produced full models containing covariates to investigate whether any difference between treatments was solely due to variation between treatments of other correlated variables. All analyses for the carcass decomposition experiment contained treatment (fresh or decomposed carcass) and all analyses for the carcass size experiment contained treatment (carcass size), as these were the focus of the experimental manipulation (Tables 3.1 and 3.2). The models for laying skew and laying spread included clutch size because it may take longer to lay larger clutches. I also included the delay until the first egg was laid in the models for laying skew because starting to lay sooner after encountering the carcass might affect laying patterns. In the models for the time until dispersal, I included laying spread because larvae in synchronous broods disperse sooner after arriving at the carcass than those in asynchronous broods (Ford and Smiseth, 2016) and clutch size because larger broods may use up the resource more quickly and therefore disperse sooner. The models for the absolute number of larvae dispersing and the proportion of eggs producing larvae that survived to dispersal included laying spread and the delay until the first egg was laid to test for a cost of asynchronous laying or starting to lay sooner after encountering the carcass. I also included clutch size in the models for the number of larvae dispersing because the number of dispersing larvae is limited by the number of eggs. I included clutch size in the models for the proportion of eggs producing larvae that survived to dispersal because females usually lay a greater number of eggs than the number of larvae the carcass can support so in large clutches a smaller proportion of the clutch may go on to produce dispersing larvae even if the viability of the eggs does not differ. The models for the mean larval mass at dispersal (excluding broods for which larvae hatched but no larvae survived to dispersal) included laying spread and the delay until the first egg was laid to test for a cost of asynchronous hatching or starting to lay sooner and the number of larvae dispersing in case there was a trade-off between number and size of larvae (Smiseth *et al*., 2014).

To determine whether egg size varied with laying order in the carcass decomposition experiment, I constructed a general linear mixed model using the

restricted maximum likelihood method including laying spread, delay until the first egg was laid, stage (first, middle, or last eggs) and treatment (fresh or decomposed carcasses) and the interaction between stage and treatment as fixed effects and brood as a random effect in the lme4 package (Bates *et al*., 2015). I carried out stepwise model reduction using likelihood ratio tests.

## 3.3 RESULTS

### 3.3.1 *Are the assumptions of the hurry-up hypothesis met?*

I found that larvae dispersed from the carcass earlier when females commenced oviposition sooner in the carcass decomposition experiment (Pearson's correlation $t_{61} = 3.99$, $P = 0.0002$; Figure 3.1a). However, there was no evidence that larvae dispersed from the carcass earlier when females commenced oviposition sooner in the carcass size experiment (Pearson's correlation $t_{67} = 0.055$, $P = 0.956$; Figure 3.1b). Thus, I found some evidence that females can shorten the time to offspring independence by commencing oviposition sooner but this response was conditional upon the specific environmental conditions of the two experiments.

As expected if starting to lay sooner after encountering the carcass leads to greater laying asynchrony, I found that laying spread was greater when females commenced egg laying earlier in the carcass size experiment (Pearson's correlation $t_{80} = -2.27$, $P = 0.026$; Figure 3.1d) and for females breeding on fresh carcasses in the carcass decomposition experiment (Kendall's Tau correlation $z = -2.15$, $P = 0.032$). However, there was no evidence for such a trade-off for females breeding on decomposed carcasses ($z = -0.17$, $P = 0.863$; Figure 3.1c). Thus, I found some evidence for a trade-off between starting to lay earlier and laying synchronously but only when females bred on fresh carcasses.

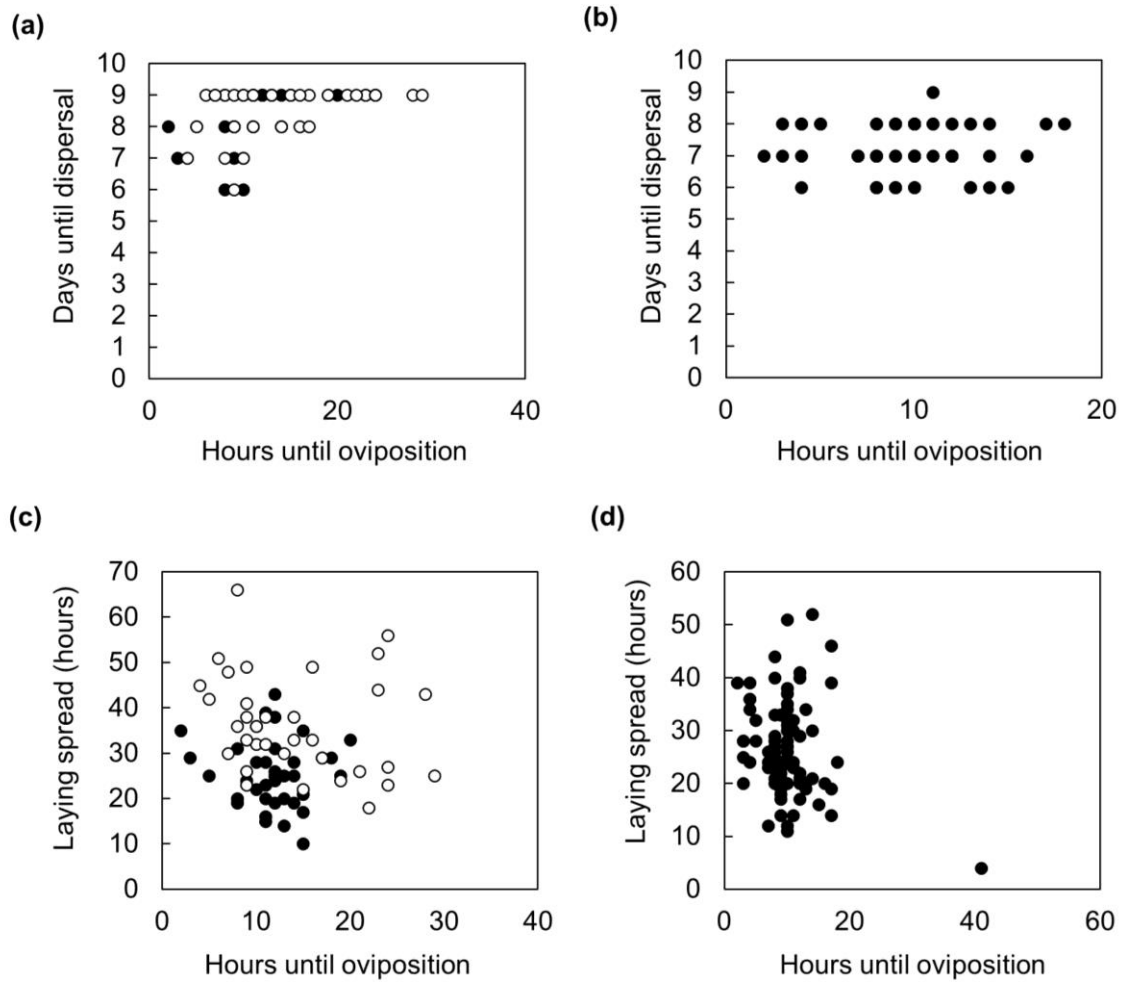



Figure 3.1: Effect of the delay until females commenced oviposition after encountering the carcass on the number of days until larval dispersal in (a) the carcass decomposition experiment and (b) the carcass size experiment, and on the laying spread (the number of hours between the first end last egg being laid) in (c) the carcass decomposition experiment and (d) the carcass size experiment. In (a) and (c), open circles represent values for females breeding on fresh carcasses and filled circles represent values for females breeding on decomposed carcasses.

Table 3.1: Outputs of models investigating female laying behaviour in response to carcass decomposition (treatment) and the consequences for the offspring. Significant effects are in bold.

| Response | Model terms | Error family | Parameter estimate (SE) | Test statistic | *P* |
|---|---|---|---|---|---|
| Delay until laying (h) | treatment | Gamma | -0.015 (0.008) | $F_{1,69} = 3.04$ | 0.0857 |
| Time until hatching (h) | treatment | Inverse Gaussian | -0.0003 (0.0004) | $F_{1,69} = 0.45$ | 0.505 |
| Time until dispersal (days) | treatment | Gaussian | 0.139 (0.219) | $F_{1,61} = 0.40$ | 0.528 |
| | clutch size | | -0.029 (0.012) | $F_{1,60} = 5.92$ | **0.018** |
| | laying spread | | 0.003 (0.011) | $F_{1,59} = 0.07$ | 0.798 |
| Laying spread (h) | treatment | Inverse Gaussian | -0.0009 (0.0002) | $F_{1,69} = 26.54$ | **<0.0001** |
| | clutch size | | <-0.0001 (<0.0001) | $F_{1,68} = 2.73$ | 0.103 |
| Laying skew | treatment | Gaussian | -0.007 (0.040) | $F_{1,69} = 0.03$ | 0.870 |
| | clutch size | | -0.0005 (0.002) | $F_{1,68} = 0.04$ | 0.837 |
| | delay until laying | | -0.004 (0.004) | $F_{1,68} = 1.39$ | 0.242 |
| Proportion of eggs surviving to dispersal | treatment | Gaussian | 0.209 (0.070) | $F_{1,69} = 8.92$ | **0.004** |
| | laying spread | | -0.009 (0.004) | $F_{1,68} = 6.07$ | **0.016** |
| | clutch size | | -0.006 (0.004) | $F_{1,67} = 2.23$ | 0.140 |
| | delay until laying | | 0.009 (0.006) | $F_{1,67} = 1.97$ | 0.165 |
| Number of larvae at dispersal | treatment | Negative binomial | 0.936 (0.220) | $\chi^2_{1,69} = 17.64$ | **<0.0001** |
| | clutch size | | 0.010 (0.013) | $\chi^2_{1,68} = 0.72$ | 0.397 |
| | laying spread | | -0.013 (0.012) | $\chi^2_{1,68} = 1.41$ | 0.235 |
| | delay until laying | | 0.004 (0.020) | $\chi^2_{1,68} = 0.05$ | 0.816 |
| Mean larval mass (g) excluding zeroes | treatment | Gaussian | 0.078 (0.008) | $F_{1,61} = 99.78$ | **<0.0001** |
| | no. of larvae | | -0.0003 (0.0003) | $F_{1,60} = 0.93$ | 0.339 |
| | laying spread | | 0.001 (0.0004) | $F_{1,60} = 1.63$ | 0.207 |
| | delay until laying | | <-0.0001 (0.0007) | $F_{1,60} = 0.004$ | 0.951 |
| Mean larval mass (g) including zeroes | treatment | Gaussian | 0.103 (0.010) | $F_{1,69} = 99.55$ | **<0.0001** |
| | no. of larvae | | 0.001 (0.0005) | $F_{1,68} = 1.21$ | 0.275 |
| | laying spread | | -0.0001 (0.0006) | $F_{1,68} = 0.06$ | 0.804 |
| | delay until laying | | -0.0007 (0.001) | $F_{1,68} = 0.55$ | 0.462 |

Table 3.2: Outputs of models investigating female laying behaviour in response to carcass size and the consequences for the offspring. Significant effects are in bold.

| Response | Model terms | Error family | Parameter estimate (SE) | Test statistic | *P* |
|---|---|---|---|---|---|
| Delay until laying (h) | carcass mass | Gamma | 0.001 (0.0007) | $F_{1,80} = 2.19$ | 0.142 |
| Time until hatching (h) | carcass mass | Inverse Gaussian | <-0.0001(<0.0001) | $F_{1,79} = 1.84$ | 0.179 |
| Time until dispersal (days) | carcass mass | Gaussian | 0.040 (0.014) | $F_{1,67} = 8.66$ | **0.004** |
| | clutch size | | 0.011 (0.013) | $F_{1,66} = 0.68$ | 0.411 |
| | laying spread | | 0.012 (0.011) | $F_{1,66} = 1.03$ | 0.314 |
| Laying spread (h) | carcass mass | Inverse Gaussian | <0.000 (<0.0001) | $F_{1,80} = 6.22$ | **0.015** |
| | clutch size | | <-0.0001 (<0.0001) | $F_{1,79} = 8.34$ | **0.005** |
| Laying skew | carcass mass | Gaussian | -0.005 (0.003) | $F_{1,80} = 3.38$ | 0.070 |
| | clutch size | | -0.003 (0.003) | $F_{1,79} = 1.52$ | 0.221 |
| | delay until laying | | 0.002 (0.004) | $F_{1,79} = 0.17$ | 0.678 |
| Proportion of eggs surviving to dispersal | carcass mass | Gaussian | 0.004 (0.004) | $F_{1,80} = 0.86$ | 0.356 |
| | clutch size | | 0.0003 (0.004) | $F_{1,79} = 0.007$ | 0.932 |
| | laying spread | | -0.005 (0.004) | $F_{1,79} = 2.14$ | 0.148 |
| | delay until laying | | -0.007 (0.006) | $F_{1,79} = 1.43$ | 0.235 |
| Number of larvae at dispersal | carcass mass | Negative binomial | 0.031 (0.015) | $\chi^2_{1,80} = 3.94$ | **0.047** |
| | clutch size | | 0.025 (0.014) | $\chi^2_{1,79} = 3.81$ | 0.051 |
| | laying spread | | -0.005 (0.012) | $\chi^2_{1,79} = 0.17$ | 0.682 |
| | delay until laying | | -0.051 (0.023) | $\chi^2_{1,79} = 2.89$ | 0.089 |
| Mean larval mass (g) excluding zeroes | carcass mass | Gaussian | 0.004 (0.0006) | $F_{1,67} = 40.05$ | **<0.0001** |
| | no. of larvae | | -0.002 (0.0004) | $F_{1,66} = 22.48$ | **<0.0001** |
| | laying spread | | -0.0005 (0.0004) | $F_{1,65} = 1.32$ | 0.255 |
| | delay until laying | | 0.001 (0.001) | $F_{1,65} = 0.91$ | 0.345 |
| Mean larval mass (g) including zeroes | carcass mass | Gaussian | 0.004 (0.001) | $F_{1,80} = 15.53$ | **0.0002** |
| | no. of larvae | | 0.002 (0.0007) | $F_{1,79} = 9.55$ | **0.003** |
| | laying spread | | 0.0002 (0.0009) | $F_{1,78} = 0.03$ | 0.863 |
| | delay until laying | | -0.0008 (0.002) | $F_{1,78} = 0.30$ | 0.588 |

3.3.2 *Do females adjust egg laying in response to environmental conditions?*
Contrary to what I predicted, there was no evidence that females commenced oviposition sooner on decomposed carcasses compared to fresh ones ($F_{1,69}$ = 3.04, $P$ = 0.086; Figure 3.2a) or as carcass size increased ($F_{1,80}$ = 2.19, $P$ = 0.142; Figure 3.2b). As a consequence, there was no difference in either the time until the first larvae hatched ($F_{1,69}$ = 0.45, $P$ = 0.505) or the time until larval dispersal between females breeding on decomposed and fresh carcasses ($F_{1,62}$ = 0.40, $P$ = 0.528). Likewise, there was no difference in the time until hatching started between females breeding on different sized carcasses ($F_{1,79}$ = 1.84, $P$ = 0.179). I found that larval dispersal was delayed as a function of increasing carcass size ($F_{1,67}$ = 8.66, $P$ = 0.004).

In contrast to what I predicted, I found that laying spread was less pronounced on decomposed carcasses than on fresh carcasses ($F_{1,69}$ = 26.54, $P$ < 0.0001; Figure 3.2c). This difference was independent of clutch size as it persisted when I controlled for the effect of clutch size ($F_{1,68}$ = 13.49, $P$ = 0.00047). Thus, females were able to lay a given clutch size more synchronously on decomposed carcasses. There was a significant increase in laying spread with carcass size ($F_{1,80}$ = 6.22, $P$ = 0.015; Figure 3.2d). However, this effect was not statistically significant when I controlled for the effects of clutch size ($F_{1,79}$ = 0.28, $P$ = 0.597). I included clutch size in the model because females laid a greater number of eggs on larger carcasses (Pearson's correlation: $t$ = 6.13, $P$ < 0.0001) and females took longer to lay larger clutches ($F_{1,79}$ = 8.34, $P$ = 0.005) on a given carcass size. Thus, the increase in laying spread on larger carcasses was likely to be solely due to the concurrent increase in clutch sizes. The laying skew index was negative for more than 90% of broods in both experiments, reflecting that the majority of the eggs were laid toward the start of the laying period. The laying skew index did not differ between females breeding on fresh and decomposed carcasses ($F_{1,69}$ = 0.03, $P$ = 0.870) and was not influenced by carcass size ($F_{1,80}$ = 3.38, $P$ = 0.070).

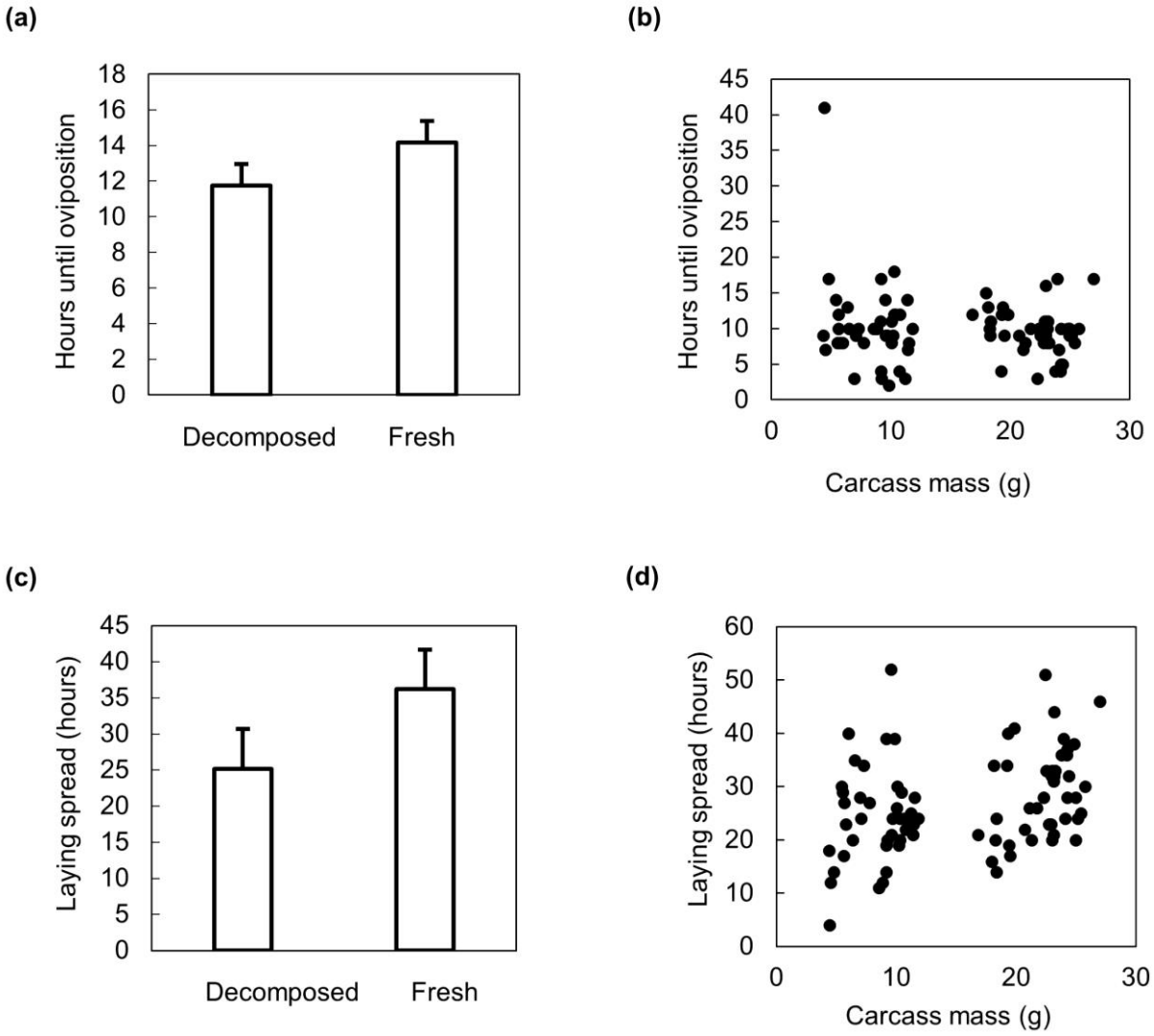



Figure 3.2: Effect of (a) carcass decomposition and (b) carcass size on the delay until females commenced oviposition after encountering the carcass and effect of (c) carcass decomposition and (d) carcass size on the laying spread (the number of hours between the first end last egg being laid). Error bars show standard errors.

3.3.3 *Does ovipositing sooner and laying asynchronously influence breeding success?*

There was no evidence that starting to lay sooner had any effects on breeding success as the time until females started laying had no significant effect on either the number of dispersing larvae (decomposition experiment $\chi^2_{1,68} = 0.05$, $P = 0.816$; carcass size experiment $\chi^2_{1,79} = 2.89$, $P = 0.089$) or the mass of these larvae (carcass decomposition experiment $F_{1,60} = 0.0038$, $P = 0.951$; carcass size experiment $F_{1,65} = 0.91$, $P = 0.345$). Not only was the proportion of eggs hatching into larvae that reached independence lower on decomposed carcasses than fresh carcasses ($F_{1,69} = 8.92$, $P = 0.004$), there was also a detrimental effect of laying asynchrony on offspring survival, with fewer offspring surviving in clutches with a greater laying spread ($F_{1,68} = 6.07$, $P = 0.016$). However, there was no significant effect of laying asynchrony on offspring survival in the carcass size experiment ($F_{1,79} = 2.14$, $P = 0.148$). Laying spread had no significant effect on the absolute number of larvae dispersing (carcass decomposition experiment $\chi^2_{1,68} = 1.413$, $P = 0.235$; carcass size experiment $\chi^2_{1,79} = 0.17$, $P = 0.682$), the time until dispersal (carcass decomposition experiment $F_{159} = 0.07$, $P = 0.798$; carcass size experiment $F_{1,66} = 1.03$, $P = 0.314$) or larval mass (carcass decomposition experiment $F_{1,60} = 1.63$, $P = 0.207$; carcass size experiment $F_{1,65} = 1.32$, $P = 0.255$).

3.3.4 *Do females lay larger eggs towards the end of the laying sequence?*

Egg volume varied across the laying sequence ($\chi^2_2 = 86.24$, $P < 0.0001$), reflecting that last-laid eggs were smaller than first-laid eggs for females breeding on both decomposed (Wilcoxon matched pairs test: $V = 602$, $P < 0.0001$) and fresh carcasses ($V = 615$, $P < 0.0001$). Furthermore, egg volume was greater for females breeding on fresh carcasses than on decomposed ones ($\chi^2_1 = 5.60$, $P = 0.018$) and there was a significant effect of the interaction between carcass decomposition and laying order on egg volume ($\chi^2_2 = 8.00$, $P = 0.018$). This interaction effect reflected that there was a greater change in egg volume across the laying order for females breeding on fresh carcasses than for females breeding on decomposed carcasses. There was no evidence that egg volume was associated with either the time until females commenced oviposition ($\chi^2_1 = 0.01$, $P = 0.945$) or laying spread ($\chi^2_1 = 1.34$, $P = 0.250$).

## 3.4 DISCUSSION

Here, I report the results of the first test of the hurry-up hypothesis in a nonavian species. This hypothesis was originally proposed as an explanation for asynchronous hatching in birds but would also apply to nonavian species, such as *N. vespilloides*, provided that the following assumptions are met: 1) starting to lay sooner shortens the time to offspring independence and 2) starting to lay sooner leads to greater laying asynchrony. My study provides some evidence that both assumptions are met in *N. vespilloides*. First, females were able to shorten the time to offspring independence as larvae dispersed earlier when females commenced oviposition sooner after encountering the carcass (though this was the case only in the carcass decomposition experiment). Second, there was evidence of a trade-off between commencing oviposition sooner and laying the eggs more synchronously (though this was not the case for females breeding on decomposed carcasses). Therefore, my results show that, as suggested by the hurry-up hypothesis, female burying beetles could shorten the time until offspring independence by starting to lay sooner after encountering the carcass, and that this in turn could lead to greater levels of laying asynchrony as a by-product. In addition to the 2 assumptions mentioned above, the following 3 predictions must also be supported for the hurry-up hypothesis to be accepted: 1) females should commence oviposition sooner when environmental conditions provide an incentive for accelerated offspring independence (such as breeding on decomposed or larger carcasses), 2) commencing oviposition sooner should have positive effects on breeding success, and 3) increased levels of hatching asynchrony should be detrimental for offspring fitness. Although multiple tests are required to test the hurry-up hypothesis, it is highly unlikely that it would be accepted due to Type 1 error given that this would require false positives for both assumptions as well as for all 3 predictions. As detailed below, I did not find sufficient evidence in support of the predictions of the hurry-up hypothesis in *N. vespilloides*. Furthermore, I urge caution when interpreting results for the covariates included in my models due to the potential for Type 1 errors associated with multiple testing.

In contrast to what I predicted, I found that females started ovipositing at a similar time after encountering the carcass regardless of whether they were breeding on decomposed or fresh carcasses. Consequently, there was no difference in the timing of larval dispersal between females breeding on decomposed and fresh carcasses. The latter result contrasts with a previous study on the same species,

which found that dispersal occurred later on decomposed carcasses than on fresh carcasses (Rozen *et al*., 2008). The different results of this previous study may reflect differences in methodology as it used experimental foster broods that were completely synchronous and smaller than my natural broods (Rozen *et al*., 2008), while I allowed females to rear their own broods without interference. I also found that females started laying at the same time after encountering a carcass regardless of its size. This result contrasts with previous work in the closely related *N. orbicollis*, which showed that larger carcasses took longer to bury and prepare and that oviposition therefore was delayed on these carcasses (Scott and Gladstein, 1993; Scott and Panaitof, 2004). This discrepancy may be due to the differences in carcass preparation and burial between the two species, reflecting that *N. orbicollis* need to bury the carcass fully beneath the soil, whereas *N. vespilloides* does not (Pukowski, 1933). I found that larvae dispersed later on larger carcasses, possibly reflecting that larvae on smaller carcasses exhausted the resource more quickly. This is consistent with previous work on *N. orbicollis*, which found that larvae on larger carcasses took longer to develop leading to later dispersal (Trumbo, 1991; Scott and Gladstein, 1993). Although there may be an incentive to accelerate offspring independence on larger carcasses due the increased risk of usurpation by other beetles and increased difficulty controlling microbial growth, larger carcasses also represent a more valuable resource that can sustain a greater number of larvae and larger larvae than smaller carcasses. These factors could interact in a complex manner to determine the optimal laying behaviour depending on the size of the carcass being used. In summary, my results provide no evidence that females shortened the time until the offspring reached independence on decomposed or larger carcasses by commencing oviposition sooner on these carcasses.

I found that there was a greater laying spread on larger carcasses but only because females laid a greater number of eggs on these carcasses and females took longer to complete larger clutches. This finding suggests that females adjust clutch size rather than hatching pattern in response to variation in carcass size and that any effect on laying spread is a by-product of the number of eggs laid. I found that females breeding on decomposed carcasses laid a given clutch size more synchronously than those breeding on fresh carcasses, suggesting that the former may attempt to lay as synchronously as possible without incurring too great a cost through a decrease in egg size or the female’s body condition. I found some

evidence of a cost of asynchronous laying in that fewer offspring survived from the egg stage until dispersal in more asynchronous broods in the carcass decomposition experiment. Previous work suggests that larvae hatching from the last-laid eggs in asynchronous clutches suffer increased mortality (Smiseth *et al*., 2008; Takata *et al*., 2014; Ford and Smiseth, 2016). When females breed on decomposed carcasses, late-laid eggs may be at a greater disadvantage due to the poor value of the carcass as a nutritional resource and reduced egg survival caused by microbes (Jacobs *et al*., 2014). Thus, under these circumstances, it may be highly advantageous for females to minimize laying spread. In contrast, it may be less advantageous for females to minimize laying spread when breeding on fresh carcasses, where moderate hatching asynchrony has a negligible effect on size or number of offspring. I found no evidence of a cost of asynchronous hatching for any aspect of offspring fitness in the carcass size experiment. The detrimental effects of hatching asynchrony may be less pronounced on larger carcasses (Müller *et al*., 1990b) because there are sufficient resources for later-hatched larvae to survive even if they are considerably smaller than their siblings. This may explain why females breeding on large carcasses do not appear to attempt to minimize laying spread in large clutches because moderate hatching asynchrony is not detrimental under these circumstances.

If females attempted to compensate for competitive asymmetries due to asynchronous hatching, I might expect an increase in egg size across the laying sequence, as reported for some birds (Schrantz, 1943; Kendeigh *et al*., 1956; Holcomb, 1969; Howe, 1976, 1978; Bryant, 1978; Hillström, 1999). Although egg size did vary with laying order, I found that egg volume decreased from first to last eggs for both fresh and decomposed carcasses, which is in the opposite direction to what I predicted. The decrease in egg size across the laying order would further disadvantage the last larvae to hatch because smaller eggs have lower survival as shown in *N. quadripunctatus* (Takata *et al*., 2015). The last offspring to hatch are out-competed by their siblings that hatched earlier and have already grown to a larger size (Smiseth *et al*., 2007a). However, due to the lesser laying spread on decomposed carcasses, competitive asymmetries would be reduced relative to fresh carcasses because the earlier larvae will have had less of a head start. Laying the clutch as synchronously as possible when breeding on a decomposed carcass may therefore reduce competitive asymmetries to a greater extent than adjusting egg size. Thus, females may favour completion of laying as

soon as possible over increasing egg volume across the clutch as a means to compensate for competitive asymmetries due to asynchronous hatching.

Overall, my results are not consistent with the hurry-up hypothesis given that females do not attempt to accelerate offspring independence under conditions where this would be favourable, such as when breeding on large carcasses or carcasses that have already started to decompose. Given that females breeding on decomposed carcasses laid more synchronously than those breeding on fresh carcasses, my results also show that females detect cues about the state of the carcass and adjust their laying spread accordingly. Additionally, my results show that there is a survival cost to offspring in asynchronous clutches in the carcass decomposition experiment while there are no detectable benefits of asynchronous laying in any treatment. It is therefore unclear why hatching asynchrony occurs given that females are capable of laying more synchronously. Asynchronous hatching has evolved independently in many different taxa including insects, reptiles, fish, and birds. However, no consensus has yet been reached on how asynchronous hatching evolves in any system. Several hypotheses have been proposed to explain the occurrence of asynchronous hatching in birds, many of which are based on constraints on the onset of incubation or adaptive incubation patterns. These hypotheses do not apply to *N. vespilloides* because it does not incubate its eggs. Previous work on *N. vespilloides* has found no evidence for the peak load reduction (Smiseth and Morgan, 2009) and sexual conflict hypotheses (Ford and Smiseth, 2016). Thus, the only remaining hypotheses are the brood reduction (Lack, 1947, 1954) and insurance hypotheses (Stinson, 1979). It seems unlikely that the original version of the brood reduction hypothesis (Lack, 1947, 1954) would apply to *N. vespilloides* because brood reduction in this species occurs through filial cannibalism rather than sibling competition (Bartlett, 1987). Nevertheless, late-hatched larvae grow less well and beg more (Smiseth *et al*., 2008), and are at a higher risk of being the victim of filial cannibalism (Andrews and Smiseth, 2013; Takata *et al*., 2013). Thus, a modified version of this hypothesis may apply to burying beetles if asynchronous hatching somehow facilitates brood reduction through filial cannibalism. The insurance hypothesis (Stinson, 1979) suggests asynchronous hatching serves as insurance against mortality of core offspring. This hypothesis may apply to burying beetles because many eggs fail to hatch and females may produce additional eggs as insurance (Bartlett, 1987). The hypothesis proposes that late-hatched marginal offspring normally only survive if

core offspring fail to hatch or die soon after hatching. The brood reduction hypothesis and insurance hypothesis may interact because, if all core offspring hatch, asynchronous hatching may provide an efficient mechanism for brood reduction (Forbes, 1990). Further studies on asynchronous hatching in my system and other nonavian systems should therefore focus on these hypotheses.

# Chapter 4

## Maternal and offspring inbreeding

This chapter has been published as referenced below, and this publication appears as Appendix E in this thesis:

Ford, L.E., Henderson, K.J. & Smiseth, P.T. 2018. Differential effects of offspring and maternal inbreeding on egg laying and offspring performance in the burying beetle *Nicrophorus vespilloides. J. Evol. Biol.* **31**: 1047-1057.

**ABSTRACT**

I investigated the effect of offspring and maternal inbreeding on maternal and offspring traits associated with early offspring fitness in the burying beetle *Nicrophorus vespilloides*. I conducted two experiments. In the first experiment, I manipulated maternal inbreeding only (keeping offspring outbred) by generating mothers that were outbred, moderately inbred or highly inbred. Meanwhile, in the second experiment, I manipulated offspring inbreeding only (keeping females outbred) by generating offspring that were outbred, moderately inbred or highly inbred. In both experiments, I monitored subsequent effects on breeding success (number of larvae), maternal traits (clutch size, delay until laying, laying skew, laying spread and egg size) and offspring traits (hatching success, larval survival, duration of larval development, and average larval mass). Maternal inbreeding reduced breeding success, and this effect was mediated through lower hatching success and greater larval mortality. Furthermore, inbred mothers produced clutches where egg laying was less skewed towards the early part of laying than outbred females. This reduction in the skew in egg laying is beneficial for larval survival, suggesting that inbred females adjusted their laying patterns facultatively, thereby partially compensating for the detrimental effects of maternal inbreeding on offspring. Finally, I found evidence of a nonlinear effect of offspring inbreeding coefficient on the number of larvae dispersing. Offspring inbreeding affected larval survival and larval development time but also unexpectedly affected maternal traits (clutch size and delay until laying), suggesting that females adjust clutch size and the delay until laying in response to being related to their mate.

## 4.1 INTRODUCTION

Inbreeding is the mating between related individuals (Wright, 1977), which often leads to a reduction in fitness referred to as inbreeding depression (Charlesworth and Charlesworth, 1987; Charlesworth and Willis, 2009). Inbreeding depression is due to a general loss of heterozygosity, which may reduce offspring fitness either by increasing the risk that recessive deleterious alleles are expressed (partial dominance hypothesis; Charlesworth and Charlesworth, 1987, 1999) or by reducing the production of heterozygotes in situations where there is heterozygote advantage (overdominance hypothesis; Charlesworth and Charlesworth, 1987, 1999). Inbreeding depression has become widely recognised as a significant evolutionary force that may drive the evolution of mate choice (Blouin and Blouin, 1988) and

mating systems (Charlesworth and Charlesworth, 1979; Lande and Schemske, 1985; Charlesworth *et al*., 1990). Furthermore, inbreeding has important implications for agriculture because it can have detrimental effects on food production (Kristensen and Sørensen, 2005) and for conservation because it can increase the risk of extinction of local populations (Crnokrak and Roff, 1999; Keller and Waller, 2002). Therefore, understanding the mechanisms and consequences of inbreeding depression is a vital subject area in evolutionary biology (Charlesworth and Charlesworth, 1987; Charlesworth and Willis, 2009).

For inbreeding depression to occur, there must be directional dominance; that is, the deleterious alleles causing inbreeding depression must be biased towards one side of the trait mean (Wright, 1977; Lynch and Walsh, 1998). When there is directional dominance, the mean value of a trait will change as a consequence of inbreeding (Lynch and Walsh, 1998). Inbreeding depression can affect traits across the entire life cycle of an organism (Charlesworth and Charlesworth, 1987). Thus, the detrimental effects of inbreeding can extend to later in life, such as when inbred individuals reproduce as adults. When this is the case, the fitness of an individual can be influenced by both its own inbreeding coefficient (offspring inbreeding) and the inbreeding coefficient of its mother (maternal inbreeding). Indeed, in some species, maternal inbreeding may have as large an influence on offspring fitness as offspring inbreeding (Mattey *et al*., 2013). Offspring and maternal inbreeding may have differential effects on traits depending on whether they are under offspring or maternal control. Traits that are expressed in the offspring, such as offspring growth and survival, can be regarded as offspring traits, while traits that are under maternal control, such as the number and size of eggs, can be regarded as maternal traits (Wilson *et al*., 2005). Offspring inbreeding is likely to influence offspring traits only given that offspring cannot influence the number and size of eggs produced by their mothers. In contrast, maternal inbreeding may affect both maternal and offspring traits, given the potential for maternal effects on offspring growth and survival (Mousseau and Fox, 1998). For example, maternal inbreeding could affect offspring growth and survival through its effects on maternally controlled traits, such as egg size, laying and hatching patterns, and maternal care (e.g., McParland *et al*., 2007). Despite their potential importance, few studies have investigated differential effects of offspring and maternal inbreeding on offspring and maternal traits associated with early offspring fitness (Walling *et al*., 2011; Mattey *et al*., 2013; Huisman *et al*., 2016). Neglecting

effects of inbreeding on these traits could lead to an underestimation of the fitness costs of inbreeding if they cause early mortality of poor quality inbred individuals.

Prior work on the effects of maternal inbreeding has focused on the offspring's performance after laying or birth, and little is known about how maternal and offspring inbreeding affects egg size (Szulkin *et al*., 2007; Fox, 2013) and patterns of egg laying or hatching. This is unfortunate given that these traits often have profound effects on the offspring's subsequent performance, thereby potentially explaining the detrimental effects of maternal inbreeding. For example, if inbred females lay smaller eggs, this can have detrimental fitness effects given that egg size often is positively associated with offspring growth and survival (birds: Krist, 2011; fish: Heath and Blouw, 1998; arthropods: Fox and Czesak, 2000). Similarly, patterns of egg laying or hatching can affect offspring survival, time to independence and size at independence (Stoleson and Beissinger, 1995; Stenning, 1996; Ford and Smiseth, 2016), thereby exacerbating the severity of inbreeding depression in offspring (de Boer *et al*., 2015). Thus, if inbred females lay clutches that hatch more asynchronously, this can also have detrimental effects on offspring performance.

Here, I investigate the effect of maternal and offspring inbreeding on breeding success and maternal and offspring traits associated with early offspring performance in the burying beetle *Nicrophorus vespilloides*. In this species, offspring suffer significant fitness costs across their life cycle when either the offspring themselves or their mothers are inbred (Mattey *et al*., 2013; Pilakouta *et al*., 2015a, 2016a; Pilakouta and Smiseth, 2016). *Nicrophorus vespilloides* is an excellent system to study the effects of inbreeding on egg size and egg laying because the eggs can be measured accurately and the timing of egg laying can be reliably recorded (Ford and Smiseth, 2016, 2017). This species breeds on small vertebrate carcasses, which are buried underground (Scott, 1998b). Females lay eggs in the surrounding soil (Pukowski, 1933) and the eggs hatch asynchronously over a period of 16–56h (Müller and Eggert, 1990; Smiseth *et al*., 2006). The larvae self-feed directly off the carcass but also beg for pre-digested carrion from the parents (Eggert *et al*., 1998). The larvae reach independence around 6 days after hatching and disperse into the soil where they pupate and ultimately eclose as adults (Smiseth *et al*., 2006). Here, I manipulate the level of inbreeding of females producing outbred offspring (maternal inbreeding) and the level of inbreeding of offspring produced by outbred mothers (offspring inbreeding). This design allows me

to test for differential effects of maternal and offspring inbreeding on maternal and offspring traits associated with early offspring performance.

It has generally been assumed that inbreeding depression is a linear function of the inbreeding coefficient (Lynch and Walsh, 1998). However, inbreeding may have nonlinear effects that would only be detected if three or more groups of individuals with different inbreeding coefficients are examined. Few studies have experimentally tested for a nonlinear effect of inbreeding, and the majority of those that have done so have focused on domestic cattle (Hudson and Van Vleck, 1984; Miglior *et al*., 1992; Biffani *et al*., 2002; Thompson *et al*., 2000; Sørensen *et al*., 2006; Croquet *et al*., 2007; Gulisija *et al*., 2007). Here I use three levels of inbreeding to test for a nonlinear relationship between the inbreeding coefficient and the magnitude of inbreeding depression: outbred (the focal individual's parents were unrelated at the grandparent level; F≈0.00), moderately inbred (the focal individual's parents were cousins; F≈0.125) and highly inbred (the focal individual's parents were siblings; F≈0.25). I measured effects of maternal and offspring inbreeding on breeding success (number of dispersing larvae), maternal traits (egg laying patterns and egg size), and offspring traits (i.e., larval survival and mass at independence). I expected both maternal and offspring inbreeding to lead to a reduction in breeding success. I expected maternal inbreeding to influence both maternal and offspring traits, given the potential for maternal effects on offspring growth and survival (Mousseau and Fox 1998). Conversely, I expected offspring inbreeding to influence offspring traits only given that offspring cannot influence the number and size of eggs produced by their mothers.

## 4.2 METHODS

### 4.2.1 Study animals

The beetles used in this study were third to sixth generation beetles from an outbred laboratory population maintained at the University of Edinburgh. The stock population descended from wild beetles caught at Corstorphine Hill, Edinburgh, U.K. (55.9500°N, 3.2833°W). In order to ensure that I had full control over the pedigree of my stock population, I housed all beetles in individual transparent plastic boxes (124mm x 82mm x 22mm) from the day that they eclosed as adults. Furthermore, when beetles were paired for breeding, I mated each female with a single male of known identity to prevent paternity uncertainty. I therefore knew the identity of the ancestors of every beetle in my laboratory population dating back to the wild-caught

beetles (Mattey and Smiseth, 2015). Keeping beetles in individual boxes from dispersal ensured that all experimental beetles were virgins at the start of the experiments. I ensured that the laboratory population was outbred by maintaining a large population each generation (86-98 broods per generation), recruiting 3 offspring only from each family to the next generation, outcrossing the laboratory population with wild-caught beetles each summer, and never mating closely related males and females (i.e., siblings or cousins; Mattey and Smiseth 2015; Mattey *et al*., 2018). These practices ensured that the inbreeding coefficient, F, in my stock population was very low (mean for individuals in the sixth generation: F = 0.0002; Mattey *et al*., 2018). Beetles were kept at 20 ± 2°C under a 16:8 light:dark cycle and were fed small pieces of organic beef twice a week. All beetles were sexually mature, virgins and of prime reproductive age (10-28 days post-eclosion) at the start of the experiments.

### 4.2.2 Experimental procedures

For the experiment on the effects of maternal inbreeding, I generated experimental females with the following three levels of inbreeding: 1) outbred females, produced by mating a given female's mother with an unrelated male (i.e., a male with whom the mother did not share a common grandparent or a closer relative; F≈0.00), 2) moderately inbred females, produced by mating a given female's mother with a cousin (i.e., a male with whom the mother shared two common grandparents; F≈0.125), and 3) highly inbred females, produced by mating a given female's mother with a brother (i.e., a male with whom the mother shared both parents; F≈0.25). I always mated experimental females to an unrelated outbred male (i.e., a male with whom the female had no shared grandparents). I did this to ensure that the offspring in this experiment always were outbred, such that any subsequent effects of the experimental treatments could be attributed to the inbreeding status of the experimental females.

For the experiment on the effects of offspring inbreeding, I generated experimental broods of offspring with the following three levels of inbreeding: 1) outbred broods, produced by mating the brood's mother with an unrelated male (i.e., a male with whom the mother did not share a common grandparent or a closer relative; F≈0.00), 2) moderately inbred broods, produced by mating the brood's mother with a cousin (i.e., a male with whom the mother shared two common grandparents; F≈0.125), and 3) highly inbred broods, produced by mating the

brood's mother with a brother (i.e., a male with whom the mother shared both parents; F≈0.25). In this experiment, I used outbred females only to ensure that any subsequent effects could be attributed to the inbreeding status of the offspring.

Different individuals were used in the maternal inbreeding and offspring inbreeding experiments. I carried out both experiments in batches of up to 20 broods at a time with the three levels of inbreeding represented in all batches to minimise any confounding effects due to potential differences between batches. At the start of the experiments, I placed the breeding female together with a male in a Petri dish (90mm x 12mm) for 4 hours to allow them time to mate (Botterill-James *et al.*, 2017). I then transferred each female to a clear breeding box (170mm x 120mm x 60mm) containing 1cm of moist compost and a previously frozen mouse carcass weighing 23.06g ± 2.26 (mean ± SD, supplied from Livefoods Direct Ltd, Sheffield, UK). The male was removed at this stage because the presence or absence of the male does not affect offspring fitness under laboratory conditions (Smiseth *et al*., 2005). In *N. vespilloides*, eggs are visible at the bottom of the breeding box and can be seen on images obtained by placing the boxes on flatbed scanners (Canon Canoscan 9000F Mark II, Canon Inc., Tokyo, Japan; Ford and Smiseth, 2016). In the small amount of soil used, the number of eggs that are visible is strongly correlated with the actual clutch size (Monteith *et al*., 2012). I scanned the breeding boxes every hour using Vuescan professional edition software (Hamrick Software, Sunny Isles Beach, FL, USA). From the scanned images, I counted the number of new eggs laid each hour to determine laying spread and laying skew (see below) and clutch size (the number of eggs laid).

In *N. vespilloides*, females determine the hatching pattern through their timing of laying (Smiseth *et al.* 2006), and I can therefore use laying time as a proxy for hatching time as the former can be determined accurately from the scans. The laying pattern can be described in two ways: the time between the first and last egg being laid, which is termed 'laying spread' (Smiseth *et al*., 2006; Takata *et al.*, 2015) and the extent to which laying is skewed towards the earlier part of the laying period, which is termed 'laying skew' (Smiseth *et al*., 2008). I calculated a laying skew index using the formula $\Sigma((t_i - t_m)/t_m)p_i$, where $p_i$ is the proportion of the total clutch that is laid in a given scan, $t_i$ is the time interval of a given scan in relation to the initiation of oviposition, and $t_m$ is the middle of the laying period (Smiseth *et al*., 2008; Ford and Smiseth, 2016). Prior work shows that this index is usually negative, indicating that egg laying is normally skewed towards the first half of the laying period. Values

closer to -1 represent a more asymmetric laying skew where a larger proportion of eggs are laid early on, while values closer to 0 represent a more symmetrical laying skew. Additionally, I measured egg size of the first five eggs that were lying flat against the bottom of the box in each clutch using ImageJ (Abramoff *et al*., 2004). Eggs were measured from the second image on which they were present given that they appear to shrink somewhat during the first hour after being laid (personal observation). For each egg, I measured its length and width three times and the mean length and width were used to calculate a prolate spheroid volume (V) using the equation $V = (1/6)\pi w^2 L$, where w is the width and L the length of the egg (Berrigan, 1991). I continued to check the scans after hatching started to gauge the number of unhatched eggs.

In order to monitor offspring performance, I checked the boxes for dispersal each day. At the time of dispersal, I counted the number of larvae in the brood and weighed the entire brood. After dispersal, I measured the pronotum width of each female with a Mitutoyo Digimatic Calliper. I confirm that there was no difference in the pronotum width of females depending on their inbreeding status (ANOVA: $F_2 = 1.00$, $P = 0.381$). I initially set up 178 pairs across my two experiments. However, 20 were excluded due to technical problems with the scanners, 23 were excluded because females did not lay any eggs, 37 were excluded from analyses of larval mass and development time because, although females laid eggs, no larvae reached dispersal and 1 was excluded because the female started laying too late to allow measurement of laying spread, laying skew or clutch size. The final sample sizes (i.e., number of pairs) in each treatment were as follows: outbred offspring $n = 21$, moderately inbred offspring $n = 24$ highly inbred offspring $n = 26$, outbred mother $n = 26$, moderately inbred mother $n = 26$, highly inbred mother $n = 28$.

### 4.2.3 Statistical analyses

I carried out the statistical analyses in R 3.3.1 (R Core Team, 2014). I first tested for effects of maternal and offspring inbreeding on breeding success; that is, the number of dispersing larvae, defined as the number of larvae in the brood at the time of dispersal. I then carried out analyses using the following nine traits (five maternal traits and four offspring traits): 1) Clutch size, defined as the total number of eggs laid before the first egg hatched (Müller *et al*., 1990b). 2) Delay until the onset of laying, defined as the number of hours after the female was placed on the carcass until the first egg was laid. 3) Laying skew index as defined above. 4)

Laying spread, defined as the number of hours between the first and last egg being laid. 5) Egg size, calculated as the average volume in $mm^3$ of five eggs in each clutch. 6) Hatching success, based on counts of the number of eggs hatching and the number of unhatched eggs. I did the analyses in two ways; that is, when including all clutches and when excluding clutches where no eggs hatched. 7) Larval survival, based on the number of larvae surviving until dispersal and the number of larvae dying between hatching and dispersal. I did the analyses in two ways; that is, when including all broods where eggs hatched and when excluding broods where eggs hatched but no larvae survived to dispersal. To ensure that all of the data were retained and the response was as informative as possible, I used the *cbind* function to bind the number of successes and failures for both hatching success and larval survival. That is, for hatching success, I used the number of eggs hatching versus the number of unhatched eggs, and for larval survival, I used the number of larvae dispersing from the carcass versus the number of larvae that died between hatching and dispersal. 8) Larval development time, calculated as the time in hours from when the first egg in the clutch was predicted to hatch until the larvae dispersed into the soil. I calculated the predicted time of hatching for the first egg by adding the average duration of egg development, which is 59 hours (Smiseth *et al.*, 2006), to the information on the onset of laying (see above). 9) Average larval mass, calculated as the mass of the brood at the time of dispersal divided by the number of larvae in the brood. I analysed data on the effects of maternal inbreeding and offspring inbreeding separately, using the same procedures for both. I carried out a separate generalised linear model (GLM) to test for an effect of maternal and offspring inbreeding status on each of the traits above. The model family used for each model is listed in tables 4.1 and 4.2. For every GLM I conducted a Fligner-Killeen test to check for homogeneity of variance. I also checked that the residuals were normally distributed, and that there were no unduly influential observations (i.e., no Cook's distances greater than 1). Because I carried out multiple tests, I applied a Benjamini-Hochberg procedure to the model results from each experiment. This did not lead to a change in the interpretation of any of the results.

## 4.3 RESULTS

### 4.3.1 Maternal inbreeding

Maternal inbreeding had a significant negative effect on breeding success, reflecting that highly inbred females produced broods with a significantly smaller number of dispersing larvae than outbred females ($\chi^2_{2,55} = 7.52$, $P = 0.023$). As expected, there were significant effects of maternal inbreeding on both maternal and offspring traits. Firstly, maternal inbreeding had a significant effect on laying skew ($F_{2,64} = 4.88$, $P = 0.011$). The majority of clutches had a negative laying skew index (92% for clutches laid by outbred females, 86% for moderately inbred females and 81% for highly inbred females), indicating that most of eggs in a given clutch were laid during the first half of the laying period. This index was closer to 0 for clutches laid by highly inbred females than for clutches produced by outbred females ($t = 3.12$, $P = 0.003$, Figure 4.1a). Thus, highly inbred females produced clutches where the eggs were laid more symmetrically around the middle of the laying period than did outbred females. There was no evidence that maternal inbreeding had a significant effect on any of the other maternal traits (Table 4.1). Secondly, maternal inbreeding had a significant effect on hatching success when I excluded broods where all eggs failed to hatch, although not when I included these broods (Table 4.1). When I excluded clutches with total hatching failure, highly inbred females produced eggs with a significantly lower hatching success than outbred females ($t = -3.64$, $P = 0.0006$, Figure 4.1b). Maternal inbreeding significantly affected larval survival when I included broods where no larvae dispersed but not when I excluded these broods (Table 4.1). When I included broods where no larvae dispersed, fewer larvae survived in broods produced by highly inbred mothers compared to broods produced by outbred mothers ($t = -2.49$, $P = 0.016$). The lack of a significant effect of maternal inbreeding on larval survival when I excluded broods where no larvae dispersed suggests that this effect was driven by a greater number of total brood failures when the mother was highly inbred (no larvae reached dispersal in 9 broods when the mother was highly inbred compared to 4 broods when the mother was moderately inbred and 4 when she was outbred). There were no significant effects of maternal inbreeding on any other offspring traits (Table 4.1). Thus, my results suggest that highly inbred females had a lower breeding success because their eggs had a lower hatching success and their larvae suffered greater mortality, rather than because they produced a smaller number of eggs.

Table 4.1: Results of GLMs testing for effects of maternal inbreeding on breeding success (number of dispersing larvae), maternal traits (clutch size, delay until laying, laying skew, laying spread and egg size) and offspring traits (hatching success, larval survival, larval development time and larval mass). Significant effects are shown in bold.

| Trait | Model error distribution | Mean outbred (± SD) | Mean moderately inbred (± SD) | Mean highly inbred (± SD) | Overall effect of maternal inbreeding | | Moderately inbred compared to outbred | | Highly inbred compared to outbred | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Test statistic | *P* | Test statistic | *P* | Test statistic | *P* |
| Number of dispersing larvae | Negative binomial | 15.47 (8.83) | 13.41 (6.73) | 8.74 (6.23) | $\chi^2_{2,55} = 7.52$ | **0.023** | $z = -0.70$ | 0.482 | $z = -2.65$ | **0.008** |
| Clutch size | Negative binomial | 30.40 (9.45) | 31.76 (8.21) | 30.81 (8.78) | $\chi^2_{2,64} = 0.27$ | 0.872 | $z = 0.52$ | 0.606 | $z = 0.16$ | 0.875 |
| Delay until laying (h) | Inverse gaussian | 18.72 (7.94) | 15.76 (7.14) | 17.76 (7.55) | $F_{2,64} = 0.74$ | 0.479 | $t = 1.32$ | 0.193 | $t = 0.40$ | 0.693 |
| Laying skew | Gaussian | -0.35 (0.22) | -0.24 (0.26) | -0.14 (0.19) | $F_{2,64} = 4.88$ | **0.011** | $t = 1.62$ | 0.110 | $t = 3.12$ | **0.003** |
| Laying spread (h) | Gaussian | 51.40 (18.53) | 51.67 (15.33) | 41.43 (17.38) | $F_{2,64} = 2.48$ | 0.092 | $t = 0.05$ | 0.958 | $t = -1.96$ | 0.055 |
| Egg size ($mm^3$) | Inverse gaussian | 1.72 (0.20) | 1.82 (0.19) | 1.77 (0.28) | $F_{2,60} = 0.91$ | 0.409 | $t = -1.34$ | 0.184 | $t = -0.65$ | 0.520 |
| Hatching success including failures | Quasibinomial | 0.77 (0.40) | 0.92 (0.11) | 0.84 (0.14) | $\chi^2_{2,64} = 4.38$ | 0.112 | $t = 1.96$ | 0.054 | $t = 0.22$ | 0.825 |
| Hatching success excluding failures | Quasibinomial | 0.96 (0.07) | 0.92 (0.11) | 0.84 (0.14) | $\chi^2_{2,59} = 15.73$ | **0.0004** | $t = -1.06$ | 0.295 | $t = -3.64$ | **0.0006** |
| Larval survival including failures | Quasibinomial | 0.38 (0.31) | 0.36 (0.27) | 0.18 (0.21) | $\chi^2_{2,59} = 8.14$ | **0.017** | $t = -0.19$ | 0.851 | $t = -2.49$ | **0.016** |
| Larval survival excluding failures | Quasibinomial | 0.47 (0.27) | 0.45 (0.22) | 0.32 (0.18) | $\chi^2_{2,42} = 3.12$ | 0.210 | $t = -0.44$ | 0.664 | $t = -1.68$ | 0.100 |
| Larval development time (h) | Inverse gaussian | 140.69 (17.82) | 142.12 (15.34) | 150.83 (11.61) | $F_{2,42} = 1.60$ | 0.213 | $t = -0.27$ | 0.789 | $t = -1.68$ | 0.101 |
| Larval mass (g) | Gaussian | 0.22 (0.06) | 0.21 (0.05) | 0.23 (0.12) | $F_{2,55} = 0.35$ | 0.707 | $t = -0.66$ | 0.513 | $t = 0.07$ | 0.944 |

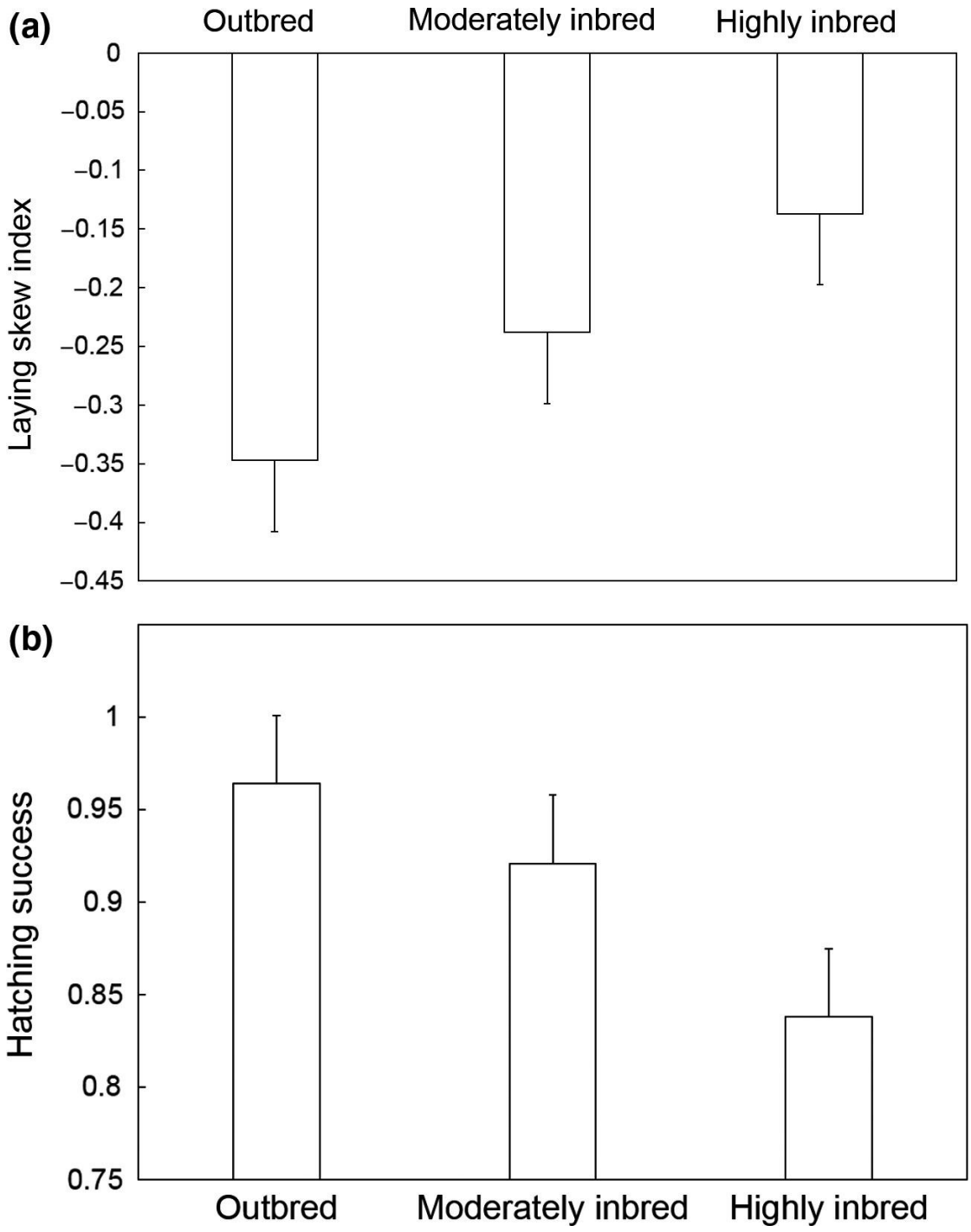



Figure 4.1: The effect of maternal inbreeding on (a) laying skew index (an index calculated based on the extent to which laying is skewed towards the earlier part of the laying period) and (b) hatching success (the proportion of the clutch that hatched, excluding clutches where eggs were laid but none hatched). Error bars represent standard errors.

### 4.3.2 Offspring inbreeding

Although there was no overall significant effect of offspring inbreeding on breeding success, there were a greater number of dispersing larvae when offspring were moderately inbred than when they were outbred ($z$ = 1.97, $P$ = 0.049). Contrary to what I expected, there were significant effects of offspring inbreeding on both maternal and offspring traits. Firstly, offspring inbreeding had a significant effect on clutch size ($\chi^2_{2,64}$ = 6.10, $P$ = 0.047). Females that were mated to an unrelated male (i.e., females producing outbred eggs) laid a greater number of eggs than females that were mated to their brother or cousin (i.e., females producing highly inbred and moderately inbred eggs, respectively) (Table 4.2). Likewise, females that were mated to their brother or cousin took on average longer to begin laying eggs than females that were mated to an unrelated male (Table 4.2). Given that offspring cannot influence the number and size of eggs produced by their mothers, these findings suggest that females adjust decisions about how many eggs to lay and when to start laying based on whether they mated with a related or an unrelated male. There was no significant effect of offspring inbreeding on any of the other maternal traits (Table 4.2). Secondly, offspring inbreeding had a significant effect on two offspring traits: larval survival from hatching to dispersal and larval development time (Table 4.2). Moderately inbred larvae had higher survival than outbred larvae when I excluded broods where no larvae dispersed ($t$ = 2.34, $P$ = 0.024, Figure 4.2a). Furthermore, moderately and highly inbred larvae developed significantly faster than outbred larvae (Table 4.2, Figure 4.2b). There was no significant effect of offspring inbreeding on hatching success (Table 4.2). Thus, keeping in mind that females mated to an unrelated male (i.e., females producing outbred eggs) laid a greater number of eggs than females mated to a related male, my results suggest that the greater breeding success when offspring were moderately inbred was associated with high larval survival from hatching to dispersal.

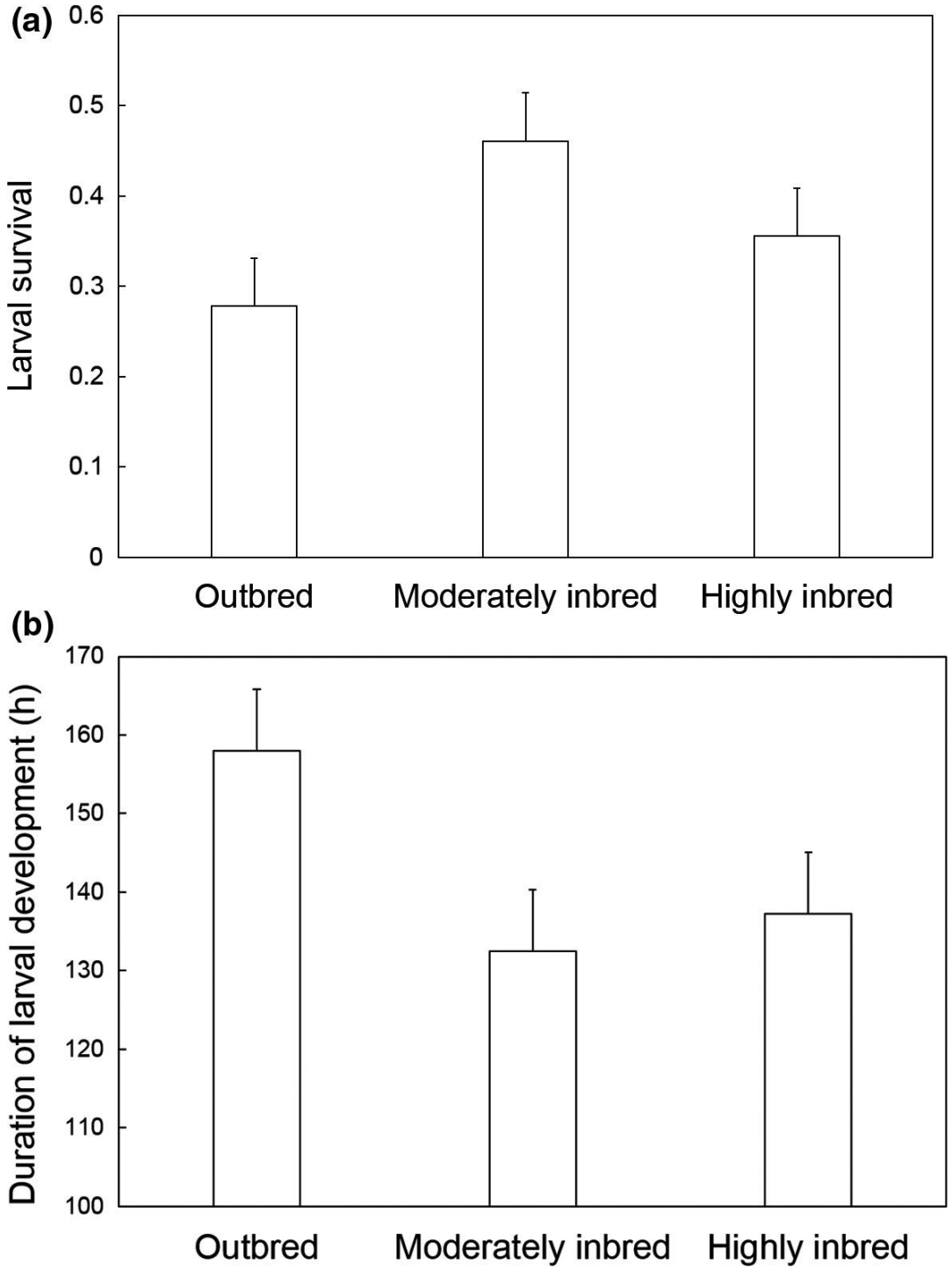



Figure 4.2: The effect of offspring inbreeding status on (a) larval survival from hatching to dispersal (the proportion of the larvae hatching that survived to dispersal, excluding broods where larvae hatched but none dispersed) and (b) the duration of larval development (the number of hours from the estimated timing of the start of hatching until dispersal). Error bars represent standard errors.

Table 4.2: Results of GLMs testing for effects of offspring inbreeding on breeding success (number of dispersing larvae), maternal traits (clutch size, delay until laying, laying skew, laying spread and egg size) and offspring traits (hatching success, larval survival, larval development time and larval mass). Significant effects are shown in bold.

| Trait | Model error distribution | Mean outbred (± SD) | Mean moderately inbred (± SD) | Mean highly inbred (± SD) | Overall effect of offspring inbreeding | | Moderately inbred compared to outbred | | Highly inbred compared to outbred | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Test statistic | *P* | Test statistic | *P* | Test statistic | *P* |
| Number of dispersing larvae | Negative binomial | 7.94 (6.18) | 12.47 (8.15) | 8.89 (5.67) | $\chi^2_{2,51} = 4.33$ | 0.115 | $z = 1.97$ | **0.049** | $z = 0.50$ | 0.618 |
| Clutch size | Negative binomial | 37.94 (15.16) | 31.04 (9.48) | 31.08 (7.83) | $\chi^2_{2,64} = 6.10$ | **0.047** | $z = -2.17$ | **0.030** | $z = -2.17$ | **0.030** |
| Delay until laying (h) | Inverse gaussian | 19.44 (8.37) | 26.63 (23.65) | 32.92 (23.99) | $F_{2,64} = 3.04$ | 0.055 | $t = -1.78$ | 0.080 | $t = -2.23$ | **0.029** |
| Laying skew | Gaussian | -0.12 (0.26) | -0.17 (0.21) | -0.18 (0.28) | $F_{2,63} = 0.36$ | 0.696 | $t = -0.67$ | 0.504 | $t = -0.82$ | 0.418 |
| Laying spread (h) | Inverse Gaussian | 45.17 (27.39) | 39.42 (16.56) | 35.92 (16.94) | $F_{2,64} = 1.08$ | 0.347 | $t = 0.87$ | 0.389 | $t = 1.51$ | 0.137 |
| Egg size ($mm^3$) | Inverse Gaussian | 1.94 (0.30) | 1.97 (0.27) | 2.03 (0.34) | $F_{2,61} = 0.42$ | 0.660 | $t = -0.27$ | 0.789 | $t = -0.85$ | 0.398 |
| Hatching success including failures | Quasibinomial | 0.85 (0.25) | 0.89 (0.17) | 0.83 (0.20) | $\chi^2_{2,64} = 0.61$ | 0.737 | $t = 0.66$ | 0.515 | $t = -0.03$ | 0.980 |
| Hatching success excluding failures | Quasibinomial | 0.90 (0.14) | 0.89 (0.17) | 0.83 (0.20) | $\chi^2_{2,63} = 1.64$ | 0.441 | $t = -0.45$ | 0.657 | $t = -1.24$ | 0.220 |
| Larval survival including failures | Quasibinomial | 0.25 (0.24) | 0.33 (0.30) | 0.24 (0.27) | $\chi^2_{2,63} = 2.26$ | 0.323 | $t = 1.46$ | 0.148 | $t = 0.67$ | 0.508 |
| Larval survival excluding failures | Quasibinomial | 0.28 (0.24) | 0.46 (0.25) | 0.36 (0.26) | $\chi^2_{2,47} = 6.00$ | **0.049** | $t = 2.34$ | **0.024** | $t = 0.82$ | 0.415 |
| Larval development time (h) | Gaussian | 158.00 (37.86) | 132.47 (28.68) | 137.28 (17.60) | $F_{2,47} = 3.53$ | **0.037** | $t = -2.52$ | **0.015** | $t = -2.07$ | **0.044** |
| Larval mass (g) | Gaussian | 0.23 (0.03) | 0.21 (0.03) | 0.20 (0.04) | $F_{2,50} = 1.86$ | 0.166 | $t = -1.06$ | 0.292 | $t = -1.93$ | 0.060 |

## 4.4 DISCUSSION

I investigated the effects of both maternal and offspring inbreeding using an experimental design with three levels of maternal and offspring inbreeding (i.e., F≈0.00, F≈0.125 and F≈0.25, respectively). Firstly, I find evidence that both maternal inbreeding and offspring inbreeding affected breeding success. As expected, highly inbred females produced broods with significantly fewer dispersing larvae than outbred females. In contrast, there was no overall significant effect of offspring inbreeding on breeding success, although there were a greater number of dispersing larvae when offspring were moderately inbred than when they were outbred. Secondly, I find evidence for differential effects of maternal inbreeding and offspring inbreeding on maternal and offspring traits. Maternal inbreeding affected laying skew, hatching success and larval survival, whereas offspring inbreeding affected clutch size, delay until the onset of egg laying, larval survival and larval development time. My results confirm that maternal inbreeding affected both maternal and offspring traits, which is in line with my expectations given that female parents can influence offspring traits through maternal effects. However, my results suggest that offspring inbreeding also affected both maternal and offspring traits, which is surprising given that offspring cannot influence the number of eggs produced by their mothers or the timing of egg laying. Thus, this finding suggests that females alter their decisions about the number of eggs they lay and the timing of egg laying based on whether they are mated with a related or an unrelated male. Finally, I found evidence of a nonlinear effect of the level of offspring inbreeding on larval survival, with moderately inbred offspring experiencing the greatest survival from hatching to independence. Below I discuss the wider implications of these results for our understanding of the effects of maternal and offspring inbreeding on breeding success and offspring performance.

I found that maternal inbreeding had a negative effect on breeding success with highly inbred females producing fewer dispersing larvae than outbred females. This finding is largely consistent with prior work reporting negative effects of maternal inbreeding on breeding success and early offspring performance in *N. vespilloides* (Mattey *et al*., 2013) as well as in birds (Keller, 1998; Reid *et al*., 2003a; Jamieson *et al*., 2003; Richardson *et al*., 2004; Szulkin *et al*., 2007) and mammals (Huisman *et al*., 2016). I recorded effects of maternal inbreeding on maternal and offspring traits associated with early offspring performance, which allowed me to identify at least some of the potential mechanisms by which maternal inbreeding

causes a reduction in breeding success. I found that maternal inbreeding affected laying skew, hatching success and larval survival. As discussed below, highly inbred females produced clutches that had a laying skew index that was closer to 0 (i.e., egg laying was less positively skewed towards the beginning of the laying period) than did outbred females. I also found that a laying skew index closer to 0 was associated with higher offspring survival, suggesting that the effect of maternal inbreeding on hatching skew cannot account for lower breeding success of highly inbred females. I found that highly inbred females laid eggs that had a significantly lower hatching success compared to eggs laid by outbred females when I excluded clutches where no eggs hatched. Furthermore, fewer larvae survived from hatching to dispersal in broods produced by highly inbred females compared to those produced by outbred females. In contrast, there was no evidence that maternal inbreeding affected clutch size, delay until laying, laying spread, egg size, larval growth or larval development time. Thus, my results suggest that the detrimental effects of maternal inbreeding on breeding success are mediated through a reduction in hatching success of eggs laid by highly inbred females and greater mortality of larvae produced by highly inbred mothers rather than a reduction in clutch size or egg size.

As mentioned above, I found that highly inbred females produced clutches that had a hatching skew index that was closer to 0 than outbred females. In other words, highly inbred females produced clutches where egg laying was less positively skewed towards the beginning of the laying period than did outbred females. This finding is contrary to what I anticipated if the detrimental effects of maternal inbreeding on breeding success were mediated through an effect on hatching skew. The reason for this is that a hatching skew index closer to 0 is assumed to be associated with increased rather than reduced offspring survival. I conducted a posthoc test of this assumption using data from the experiment on the effects of offspring inbreeding (I used these data because all mothers were outbred). I found that a more negative value of laying skew index (i.e., when egg laying was more strongly positively skewed towards the beginning of the laying period) was associated with reduced larval survival (Pearson's correlation, $t_{46} = 3.07$, $P = 0.004$, $r = 0.41$) as well as with fewer dispersing larvae ($t_{47} = 2.74$, $P = 0.009$, $r = 0.37$). This effect is presumably mediated through an effect of laying skew on sibling competition. There is good evidence from studies on birds that asynchronous hatching is associated with asymmetric sibling competition (Magrath, 1990; Stoleson

and Beissinger, 1995; Mock and Parker, 1997), where early-hatched offspring are competitively superior to offspring that hatch later (Lack, 1947; Stinson, 1979). Likewise, studies on *N. vespilloides* and the closely related *N. quadripunctatus* find that late-hatched larvae have reduced growth rates and are less likely to survive to independence than early-hatched larvae (Smiseth *et al*., 2007a; Takata *et al*., 2013). Thus, the finding that highly inbred females produce clutches where egg laying was less positively skewed towards the beginning of the laying period suggests that these females adjust their laying patterns facultatively, and that by doing so, they partially mitigate some of the negative effects of maternal inbreeding on breeding success. However, I note that females cannot completely compensate for the detrimental effect of maternal inbreeding given that the number of larvae reaching dispersal is still lower for broods produced by highly inbred females.

Although there was no overall significant effect of offspring inbreeding on breeding success, I found that a greater number of larvae reached dispersal in broods where offspring were moderately inbred compared to broods where they were outbred. I also found that females that were mated to related males (and thus were producing inbred offspring) laid fewer eggs than those that were mated to unrelated males (and thus producing outbred offspring). This finding is consistent with a recent theoretical model predicting that parents producing inbred offspring should produce fewer offspring and invest more resources in individual offspring (Duthie *et al*., 2016). Additionally, I found that females producing highly inbred offspring took longer to begin egg laying after encountering the carcass. It is unlikely that this would be beneficial for the offspring given that this delay would be associated with an increase in microbial growth over time, which reduces egg survival (Jacobs *et al*., 2014). I found that inbred larvae developed more quickly from hatching to dispersal than outbred larvae but attained a similar average mass at dispersal. Highly inbred larvae dispersed around 21 hours sooner after hatching than outbred larvae, which more than counteracted the 13-hour delay in the onset of laying by females producing highly inbred offspring. My study provides no information on the mechanism behind the shorter development time for highly inbred larvae. However, a recent study on the same species found that outbred females provide more direct care towards inbred larvae, resulting in inbred and outbred larvae attaining the same mass at dispersal (Mattey *et al*., 2018). Thus, it is possible that an increase in direct care towards inbred larvae allows them to develop faster (Lock *et al*., 2004), which would be beneficial for the offspring because the carcass

becomes increasingly inhospitable for larvae as decomposition occurs (Rozen *et al*., 2008). If so, this could contribute to the greater larval survival that I observed in moderately inbred broods, leading to a greater number of larvae at dispersal.

Finally, my results provide evidence of a nonlinear effect of offspring inbreeding on breeding success. I found that a greater number of larvae reached dispersal in broods of moderately inbred larvae compared to broods of either outbred or highly inbred larvae. Likewise, moderately inbred larvae had greater survival than either outbred or highly inbred larvae when excluding broods where no larvae dispersed. Previous studies on this species have found evidence for an effect of offspring inbreeding on offspring survival (Mattey *et al*., 2013; Pilakouta *et al*., 2015a, 2016a; Pilakouta and Smiseth, 2016). I note that these studies also found detrimental effects of offspring inbreeding on other components of offspring performance, such as survival from dispersal to eclosion (Mattey *et al*., 2013; Pilakouta *et al*., 2015a, 2016a, Pilakouta and Smiseth, 2016), survival from hatching to eclosion (Pilakouta *et al*., 2016a) and adult lifespan (Pilakouta *et al*., 2015a; Pilakouta and Smiseth, 2016). My design differs from that used in the majority of laboratory studies investigating inbreeding depression, which simply compare fitness-related traits of outbred individuals with experimentally generated inbred individuals (with the inbreeding coefficient of the inbred treatment varying between studies) (Lynch and Walsh, 1998). Meanwhile, studies on inbreeding depression in the field often determine inbreeding coefficients from a pedigree, often assuming that inbreeding depression is a linear function of the inbreeding coefficient (Lynch and Walsh, 1998). Nevertheless, there is some evidence for nonlinear effects of inbreeding from studies on domestic cattle (Hudson and Van Vleck, 1984; Miglior *et al*., 1992; Biffani *et al*., 2002; Thompson *et al*., 2000; Sørensen *et al*., 2006; Croquet *et al*., 2007; Gulisija *et al*., 2007), and studies on mice find that offspring produced by intermediately related parents are larger than those produced by unrelated or closely related parents (Barnard and Fitzsimons 1989; Keane 1990). There is also some evidence from studies on humans that couples that are moderately related to each other have a greater number of children (Helgason *et al*., 2008; Labouriau and Amorim, 2008). I suggest that such non-linear effects of offspring inbreeding could arise as a consequence of maternal effects on offspring. There is evidence that maternal care buffers against the detrimental effect of offspring inbreeding in *N. vespilloides* (Pilakouta *et al*., 2015a). Furthermore, the smaller clutch sizes laid by females producing inbred offspring may lead to a lower initial number of larvae

hatching, which would allow females to provide more care to each larva in inbred broods as predicted by recent theoretical model (Duthie *et al*., 2016). Thus, non-linear effects of inbreeding could arise if females overcompensate for the detrimental effects of moderate offspring inbreeding by providing more care, resulting in higher larval survival for moderately inbred offspring compared to outbred offspring. Meanwhile, an increase in maternal care may just be sufficient to mitigate the detrimental effects of inbreeding when offspring are highly inbred, resulting in similar survival of highly inbred and outbred offspring (Mattey *et al.*, 2018).

In conclusion, I found that both maternal inbreeding and offspring inbreeding affected breeding success, that maternal inbreeding and offspring inbreeding affected different traits associated with early offspring performance, and that there were non-linear effects of offspring inbreeding. My results provide novel insights into inbreeding by suggesting that maternal inbreeding and offspring inbreeding have differential effects on maternal and offspring traits. Maternal inbreeding affected laying skew, hatching success and larval survival; whereas offspring inbreeding affected clutch size, delay until onset of egg laying, larval survival and larval development time. Furthermore, my results suggest that inbred females facultatively adjust their laying patterns to compensate for some of the detrimental effects of maternal inbreeding on offspring. In support of this, I found that inbred females lay clutches with hatching skew index that was closer to 0 (i.e., the eggs were laid more symmetrically around the middle of the laying period), which is associated with greater offspring survival. Finally, I found evidence of a nonlinear effect of offspring inbreeding coefficient on the number of larvae dispersing, with the greatest number of larvae dispersing in moderately inbred broods, reflecting that these broods experienced the greatest larval survival. This result highlights the importance of considering deviations from linearity when testing for an effect of inbreeding and I recommend that future studies incorporate multiple inbreeding treatments where possible in order to increase our understanding of the effects of inbreeding and to gain an insight into the potential mechanisms behind these effects.

# Chapter 5

# Effects of age on within-brood variation in hatching times

**ABSTRACT**

Evolutionary theory predicts that maternal effects should senesce, and within-brood distributions of traits associated with maternal effects may also change with maternal age in addition to any changes in mean trait values. I investigated the effect of female age on higher moments of within-brood distributions of hatching times in *N. vespilloides*, using laying time as a proxy for hatching time. The relative hatching times of larvae in the brood affect offspring fitness because hatching asynchrony leads to asymmetric sibling competition. If selection is relaxed at older ages I expected that older females would produce broods with later hatching, and a greater variance and more extreme skew in hatching times. Females breeding at older ages did lay later. However, they produced clutches that had a smaller variance in laying times and were less positively skewed (laying was less skewed towards the start of the laying period). There was a trend towards decreased clutch size with age and this was associated with later laying, reduced within-clutch variance in laying times and a less positively skewed distribution of laying times. There was evidence for selective disappearance of less fecund females.

## 5.1 INTRODUCTION

Senescence is the age-related deterioration of organismal function and traits associated with fitness. It is believed to be caused ultimately by age-related reductions in the strength of selection for survival and reproduction (Medawar, 1952; Williams, 1957; Hamilton, 1966). Most formal theory explicitly addresses ageing manifested as declines in survival and reproductive rates with increased age (Hamilton, 1966; Charlesworth, 1994, 2001), and this sort of ageing is the primary focus of most studies (e.g., Jones *et al*., 2014). However, fitness traits associated with maternal effects, such as offspring birth weight in Soay sheep (Hayward *et al*., 2013) and egg quality in blue-footed booby (Beamonte-Barrientos *et al*., 2010), also show declines with maternal age. Consistent with these observations, recent evolutionary theory predicts that maternal effects should rapidly senesce (Moorad and Nussey, 2016). Studies on the effects of maternal age on offspring traits have focused on the effects of age on central tendencies of population distributions. For example, increasing maternal age is associated with reduced mean egg size in the fruit fly *Drosophila melanogaster* (Moore and Harris, 2003) and reduced mean amounts of nutrients deposited in eggs in the parasitic wasp *Eupelmus vuilleti* (Muller *et al*., 2017).

In species that produce multiple offspring in each reproductive bout, however, females make repeated decisions regarding offspring traits, such as the allocation of nutrients to each egg and the timing of hatching or birth of each offspring. These reproductive decisions may introduce variation between offspring within a brood or litter for traits associated with maternal effects. If maternal effects change with age, then the magnitude of within-brood variance may also change with age in addition to any changes in mean trait values. For example, within-clutch variability in maternal allocation of resources to each egg appears to decrease with maternal age in the soil mite *Sancassania berlesei* while mean egg size increases (Crean and Marshall, 2009). Evolutionary theory predicts an increase in within-population additive genetic variances of mortality and fertility (Charlesworth, 2001; Moorad and Promislow, 2009), but this is a fundamentally different phenomenon as this contributes to among-individual variance at the level of the population. Maternal age effects on within-brood variance have been overlooked when developing theoretical models and in experimental studies of senescence. There is now a need to rectify this because traits associated with maternal effects can vary between siblings and can profoundly affect offspring fitness (e.g., Rossiter, 1991; Bosman, 2014), and it is crucial to investigate how these traits might senesce to gain a more comprehensive understanding of the evolution of ageing.

Within-brood variation in hatching times is particularly important because it can have a greater effect on offspring performance than other maternally-controlled traits, such as egg size (Bitton *et al*., 2006). Asynchronous hatching (where offspring from a single reproductive event hatch over an extended period of time) is a major determinant of within-brood variation in growth and survival across many different taxa (Lack, 1954; Gilmore, 1993; Stoleson and Beissinger, 1995; Nalepa, 1998; Ryan and Plague, 2004; Smiseth *et al*., 2006; While *et al*., 2007). It is likely that offspring that hatch (or are born) very early or very late have lower fitness (e.g. Thomas *et al*., 2001; Shine and Olsson, 2003; Visser *et al*., 2011; Lof *et al*., 2012; Jacobs *et al*., 2014). Additionally, when siblings interact after hatching, as in species where parents provision them with food, their relative hatching times will affect offspring fitness because hatching asynchrony leads to asymmetric sibling competition (Mock, 1984; Magrath, 1990; Stoleson and Beissinger, 1995; Mock and Parker, 1997; Parker *et al*., 1989). Offspring that hatch later are competitively disadvantaged compared to their siblings that hatched earlier (Howe, 1976; O'Connor, 1978; Clark and Wilson, 1981; Mock, 1984). Thus, the distribution of

hatching times within a brood can affect survival rates and mean offspring size (Hahn, 1981; Magrath, 1989; Hébert, 1993; Smiseth *et al*., 2008; Smiseth and Morgan, 2009; Ford and Smiseth, 2016). Consequently, females may be under selection to produce a hatching pattern that features a particular amount of within-brood variance. If this is the case, I might expect to observe a change in the amount of within-brood variation (or higher moments about the mean; such as skew) in hatching times with increased maternal age if selection is relaxed (as in late age). However, I note that this verbal argument is yet to be supported by formal models for how higher moments of traits under maternal control should evolve to senesce.

Here I report on an experiment in which I investigated maternal effect senescence of hatching patterns in the burying beetle *Nicrophorus vespilloides*. Like all burying beetles within this genus, this species breeds on carcasses of small vertebrates that they bury underground (Scott, 1998b), and females lay eggs singly in the surrounding soil (Pukowski, 1933). Unlike birds, these beetles do not incubate the eggs, and the development time of eggs is therefore fairly uniform at a constant temperature. The timing of laying can be measured reliably and can be used as a proxy for hatching time because the hatching pattern is determined by the laying pattern (Smiseth *et al*., 2006). *Nicrophorus vespilloides* is an excellent model system for investigating maternal effects on higher central moments of hatching times because females lay clutches of an average of 41 eggs that hatch over a mean period of around 30 hours (Smiseth *et al*., 2006). Hatching patterns in this species affect offspring growth and survival (Smiseth *et al*., 2008; Smiseth and Morgan, 2009; Ford and Smiseth, 2016), and both of these traits should influence fitness (larger individuals are more likely to breed because they win fights against smaller individuals when competing for a carcass – see Otronen, 1988). Fewer larvae survive in experimentally generated broods where the larvae arrive at the carcass asynchronously compared to synchronously (Smiseth and Morgan, 2009; Ford and Smiseth, 2016), and the surviving larvae are smaller on average in broods hatching over a longer period of time (Ford and Smiseth, 2016). Although the larvae can feed from the carcass themselves, they can also beg for pre-digested carrion from the parents (Smiseth *et al*., 2003). Parental food provisioning increases larval growth and accelerates development (Lock *et al*., 2004). The larvae develop together on the carcass, and siblings compete for access to the parents to acquire food. Variation in hatching times influences the relative competitive abilities of larvae in the brood, which affects their growth and survival (Smiseth *et al*., 2007a). Here I

investigated maternal effect senescence of hatching patterns by allowing virgin females to breed at different ages and recording the timing of laying of each egg to test for an effect of maternal age on the means, variances, and skews of within-brood distributions of hatching times.

## 5.2 METHODS

### 5.2.1 Study animals

The beetles used in this study were from an outbred laboratory population maintained at the University of Edinburgh. These descended from wild beetles caught in Edinburgh, United Kingdom. The beetles were bred in the lab for at least two generations before the experiment. Each week, I generated experimental beetles by mating virgin males and females that were unrelated at the grandparent level. Upon reaching adulthood, the resulting offspring were housed individually in clear plastic boxes (124mm x 82mm x 22mm) filled with moist organic compost soil. They were kept at 20 ± 2°C under a 16:8 light:dark cycle and were fed small pieces of organic beef twice a week.

### 5.2.2 Experimental procedures

I paired virgin females aged 11 to 79 days post-eclosion with unrelated virgin males of prime reproductive age (10 to 26 days post-eclosion) and placed them in a clear breeding box (170mm x 120mm x 60mm) containing 1cm of moist compost. Adults reach sexual maturity around 10 days after eclosion (Cotter *et al*., 2011). Given that only 1% of virgin females survive to 77-87 days post-eclosion (Moorad unpublished), the age range used in my experiment covers almost the entire adult lifespan. I supplied each pair with a previously frozen mouse carcass (range: 19.35-26.78g; supplied from Livefoods Direct Ltd, Sheffield, UK). I initially set up 181 pairs, and 135 of these pairs produced viable eggs, resulting in a total sample size of 5000 eggs in the analyses. Eggs are visible at the bottom of the breeding box in the small amount of soil used, and the visible number of eggs is very similar to the actual clutch size (Monteith *et al*., 2012; Pearson's correlation: $r = 0.98$, $n = 21$, $P < 0.001$). I scanned the breeding boxes every hour using flat-bed scanners (Canon Canoscan 9000F Mark II, Canon Inc., Tokyo, Japan) (Ford and Smiseth, 2016) and Vuescan professional edition software (Hamrick Software, Sunny Isles Beach, FL, USA) until the eggs hatched. In accordance with convention for studies on this species, I defined a clutch as all eggs laid before the first larva hatched (Müller, 1987; Steiger,

2013). I excluded clutches in which all eggs failed to hatch because females will continue to lay eggs if larvae do not arrive at the carcass, resulting in aberrant laying patterns (Müller, 1987). From the scanned images, I counted the number of new eggs laid each hour and calculated the sum of these values to determine the clutch size.

I assigned a value $t_{ij}$ to each egg *ij* in clutch *i* representing the number of hours after the female encountered the carcass until that egg was laid. I subtracted the mean time of laying for each clutch, $t_{i.}$, from the value for each egg, and calculated the second-and third-order deviations from the clutch-mean, $(t_{ij}\text{-}t_{i.})^2$, and $(t_{ij}\text{-}t_{i.})^3$. I refer to these individual-specific laying measures as $d_1$, $d_2$, and $d_3$. The clutch-specific averages of these measures correspond to the within-clutch timing means, variances, and skews, respectively. After breeding, I measured the pronotum width of each female using a Mitutoyo Absolute Digimatic calliper (with an accuracy of ±0.02mm) and checked females at least three times a week to obtain information on their approximate age at death.

### 5.2.3 Statistical analyses

I fit my data to linear mixed models in ASReml version 4.1 (Gilmour *et al*., 2015). The fixed effects included: 1) female age at breeding; 2) clutch size, as this can influence laying patterns (Ford and Smiseth, 2017); 3) carcass size (linear and quadratic effects, using the *pol* function), as this can also influence laying behaviour (Müller *et al*., 1990b); and 4) age at death to control for any selective disappearance of poor quality individuals (van de Pol and Verhulst, 2006; Blas *et al*., 2009; Hayward *et al*. 2013) that might obscure the effect of senescence on laying patterns.

Female age at breeding was treated as a continuous linear variable, although most females were around 2, 5, 8 or 11 weeks old due to the nature of the breeding design used to generate the experimental females. Clutch size was fit as a continuous linear function. Age at death was treated as a categorical variable (Ivimey-Cook and Moorad, 2018) divided into four classes based on the experimental female ages of 2, 5, 8 or 11 weeks, such that age at death was categorised as either during the first (days 15-35 post-eclosion), second (days 36-56), third (days 57-77), or fourth class (78 days or more) and the fourth class was used as the reference value. Clutch ID was added as a random effect to avoid pseudoreplication because each clutch consisted of several eggs laid by the same female.

Using these fixed and random effects, I fit the data to a multivariate mixed model using $d_1$, $d_2$, and $d_3$ as response variables. However, this model failed to converge unless age at death was removed. I then fit three univariate models, each with $d_1$, $d_2$, or $d_3$ as the response variable and the same set of fixed and random effects as the multivariate model. In none of these models did age at death have a significant effect (Table 5.1). This justifies my decision to fit a new multivariate model that excluded of age at death as a predictor by demonstrating that this factor did not significantly influence the distribution of laying times.

To determine the significance of effects of female age and clutch size on each laying metric, I calculated *P*-values from *z*-scores obtained by dividing the estimated effect sizes for each laying metric by their estimated standard errors. Larger clutches were associated with smaller $d_1$. This could have been because higher quality females were able to produce a larger number of eggs while requiring a shorter time before laying. In this case, female quality might have been associated with two easily measured traits: body size and lifespan. To investigate this possibility, I fit a multivariate model to test whether $d_1$ was affected by female lifespan (as a continuous variable) and female pronotum width (a measure of body size), and I also included clutch ID as a random effect. I restricted the data to include only females aged between 20 and 50 days post-eclosion to reduce other sources of variation in $d_1$. Because clutch size had a significant effect on the laying metrics in the multivariate model, I investigated the indirect effects of female age on the laying metrics via its effects on clutch size by fitting a univariate mixed model with clutch size as the response variable and the same fixed and random effects as before (excepting clutch size).

Because $d_2$ and $d_3$ are the result of related operations carried out on the same initial values, I also carried out analyses at the level of the clutch rather than each egg, using mean time of laying for the clutch and the laying spread and laying skew index as used throughout this thesis (Addendum to Chapter 5).

## 5.3 RESULTS

### 5.3.1 *Direct effects of age on laying metrics*

I carried out a multivariate analysis to explain how changes in female age, clutch size and carcass size affect $d_1$, $d_2$ and $d_3$ (Table 5.2). All laying metrics decreased with female age: $d_1$ decreased by 0.11h for each additional day of female age ($z$ = -2.26, $P$ = 0.024), while $d_2$ decreased by 1.19h$^2$, ($z$ = -3.46, $P$ < 0.001) and $d_3$

decreased by 14.00h$^3$ ($z$ = -2.30, $P$ = 0.022). In other words, older females laid eggs sooner after encountering the carcass and produced clutches with less variation in the timing of laying and less positive laying skew than young females. There were also effects of clutch size on laying metrics. For each additional egg in the clutch, $d_1$ decreased by 24.09h ($z$ = -3.65, $P$ < 0.001), $d_2$ increased by 36.81h$^2$ ($z$ = 0.73, $P$ = 0.466) and $d_3$ increased by 2029.16h$^3$ ($z$ = 2.12, $P$ = 0.034). Thus, eggs were laid earlier in larger clutches, and these clutches had a greater variance and a more positively skewed distribution of laying times. Carcass size had no significant effect on the laying metrics (Table 5.2).

I fit a model to investigate whether the reduction in $d_1$ with larger clutch sizes was attributable to large body size or long lifespan of females, as both are possible indicators of higher female quality. Neither trait significantly affected laying times. There was a non-significant decrease of 0.92h/mm (SE = 0.83, $z$ = -1.11, $P$ = 0.268) and a non-significant increase of 25.39h/day (SE = 38.51, $z$ = 0.66, $P$ = 0.510).

Table 5.1: Effect of age at death in univariate models for $d_1$, $d_2$ and $d_3$.

| Age at death | $d_1$ | | | $d_2$ | | | $d_3$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | Effect size (SE) | *z* | *P* | Effect size (SE) | *z* | *P* | Effect size (SE) | *z* | *P* |
| First class | 9.652 (13.085) | 0.73 | 0.461 | 66.885 (96.573) | 0.69 | 0.489 | 1964.643 (2728.29) | 0.72 | 0.471 |
| Second class | -9.877 (12.231) | -0.81 | 0.419 | -60.153 (85.171) | -0.71 | 0.480 | 36.811 (2310.135) | 0.02 | 0.987 |
| Third class | -2.100 (3.933) | -0.53 | 0.593 | 37.480 (20.858) | 1.80 | 0.072 | 811.632 (373.735) | 2.17 | 0.030 |
| *F* test | $F_{3, 73.4} = 0.58$ | | $P = 0.630$ | $F_{3, 136.4} = 1.41$ | | $P = 0.243$ | $F_{3, 208.5} = 1.71$ | | $P = 0.167$ |

Table 5.2: Model output from a multivariate analysis investigating how changes in female age, clutch size and carcass size affect $d_1$, $d_2$ and $d_3$.

| | | $d_1$ | | | $d_2$ | | | $d_3$ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Variables | | Effect size (SE) | *z* | *P* | Effect size (SE) | *z* | *P* | Effect size (SE) | *z* | *P* | *F* test | *P* |
| Female age | | -0.106 (0.047) | -2.26 | 0.024 | -1.188 (0.344) | -3.46 | <0.001 | -14.000 (6.098) | -2.30 | 0.022 | $F_{3,120.7} = 4.49$ | 0.005 |
| Clutch size | | -24.085 (6.597) | -3.65 | <0.001 | 36.813 (50.547) | 0.73 | 0.466 | 2029.161 (955.073) | 2.12 | 0.034 | $F_{3,141.8} = 5.80$ | <0.001 |
| Carcass size | Linear | 4.998 (2.390) | 2.09 | 0.037 | -21.955 (17.469) | -1.26 | 0.209 | -704.009 (307.584) | -2.29 | 0.022 | $F_{6,152.4} = 2.03$ | 0.065 |
| | Quadratic | 0.645 (3.721) | 0.17 | 0.862 | -16.248 (27.211) | -0.60 | 0.550 | 257.885 (474.366) | 0.54 | 0.587 | | |

### 5.3.2 *Indirect effects of age on laying metrics*

The aforementioned multivariate model estimates the direct effects of female age and clutch size on the laying metrics, but it does not estimate indirect effects of age that arise through correlated effects of female age on clutch size. To investigate these effects, I carried out an additional analysis to explain how changes in female age, age at death and carcass size determine clutch size (Table 5.3). Clutch size decreased by 0.12 eggs for each additional day of female age (SE = 0.06, $z$ = -1.73, $P$ = 0.084). Thus, there was a (non-significant) trend for older females to produce smaller clutches. Carcass size had no significant effect on clutch size (Table 5.3). This model converged when age at death was used as a correlate, and I found evidence for selective disappearance of less-fertile females from the cohort in early life; females that died soonest were those that produced the smallest clutches. Those females that survived beyond the oldest age of reproduction (78 days or more post-eclosion) laid about 37 eggs on average. In comparison, females that died 15 to 35 days post-eclosion produced an average of 33.02 fewer eggs than the longest-lived females ($z$ = -3.58, $P$ < 0.001), while females that died 36 to 56 days post-eclosion produced an average of 28.33 fewer eggs ($z$ = -3.18, $P$ = 0.001) and females that died 57 to 77 days post-eclosion produced an average of 1.85 more eggs ($z$ = 0.58, $P$ = 0.565).

The indirect effects of age on the laying metrics were calculated by taking the product of this effect of age on clutch size and the effects of clutch size on laying metrics. Eggs in smaller clutches were laid later. Therefore, the decrease in clutch size with age was associated with an increase in the time until laying. As a result of the indirect effect of age on $d_1$ mediated through clutch size, there was an increase in $d_1$ of 2.80h for each additional day of female age, which opposes the direction of the decrease of 0.11h for each additional day resulting from the direct effect of female age (Figure 5.1). Smaller clutches had a smaller variance in laying times and laying times were less skewed towards the beginning of the laying period (less positively skewed). Therefore, the decrease in clutch size with age contributed to the decrease in $d_2$ and $d_3$. The indirect effect of age mediated through clutch size resulted in a decrease of 4.27h$^2$ in $d_2$, and a decrease of 235.63h$^3$ in $d_3$ for each additional day of female age, reinforcing the decrease of 1.19h$^2$ in $d_2$ (Figure 5.2) and the decrease of 14.00h$^3$ in $d_3$ (Figure 5.3) for each additional day of female age resulting from the direct effect of female age.

Table 5.3: Model output from an analysis investigating how changes in female age, age at death and carcass size determine clutch size.

| Variable | | Effect size (SE) | *z* | *P* | *F* test | *P* |
|---|---|---|---|---|---|---|
| Female age | | -0.116 (0.067) | -1.73 | 0.084 | $F_{1,65.0} = 2.98$ | 0.089 |
| Age at death | First class | -33.022 (9.231) | -3.58 | <0.001 | $F_{3,65.0} = 7.87$ | <0.001 |
| | Second class | -28.329 (8.913) | -3.18 | 0.001 | | |
| | Third class | 1.855 (3.220) | 0.58 | 0.564 | | |
| Carcass size | Linear | 4.496 (4.295) | 1.05 | 0.295 | $F_{2,65.0} = 1.91$ | 0.157 |
| | Quadratic | 5.067 (8.364) | 0.61 | 0.545 | | |

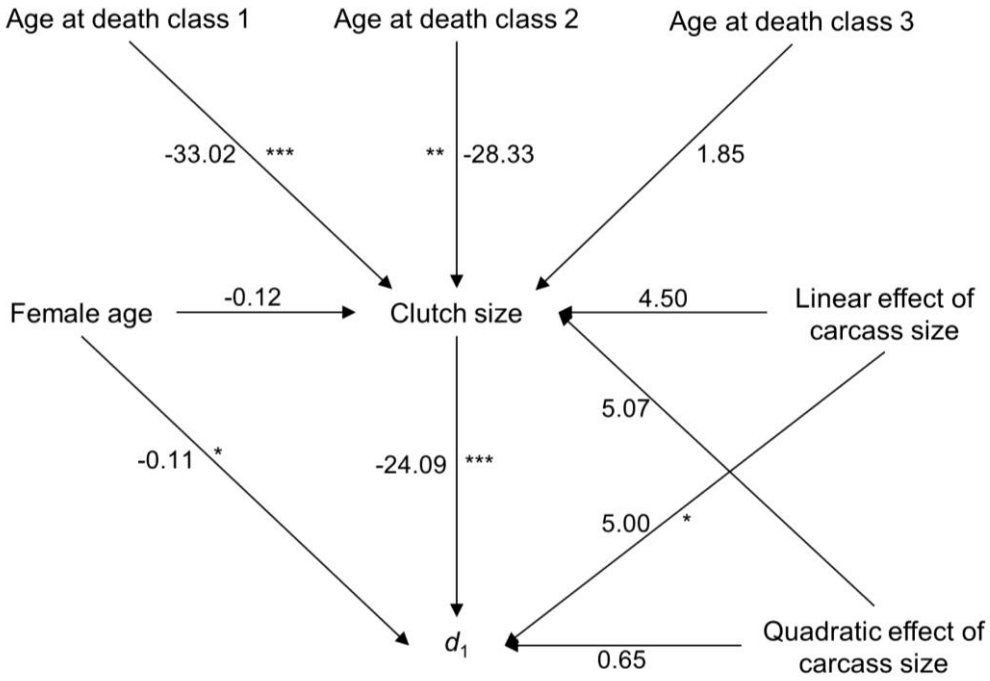



Figure 5.1: Path diagram showing the direct and indirect effects of age on $d_1$. Asterisks indicate significance (* indicates P < 0.05, ** indicates P < 0.01, *** indicates P < 0.001). The linear and quadratic effects of carcass size are defined as orthogonal and therefore no correlation between them is represented.

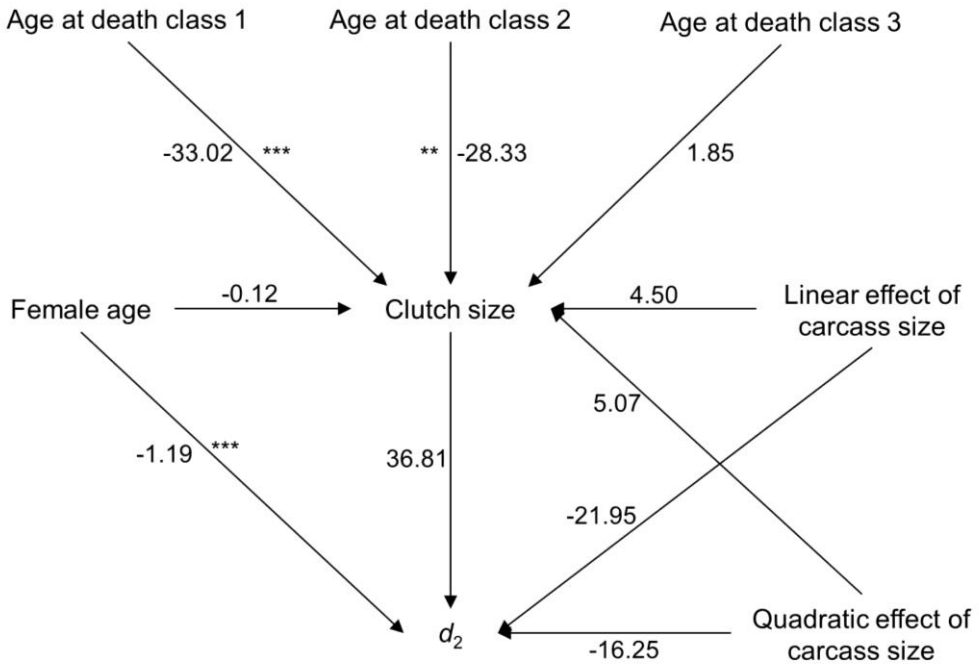



Figure 5.2: Path diagram showing the direct and indirect effects of age on $d_2$. Asterisks indicate significance (* indicates P < 0.05, ** indicates P < 0.01, *** indicates P < 0.001). The linear and quadratic effects of carcass size are defined as orthogonal and therefore no correlation between them is represented.

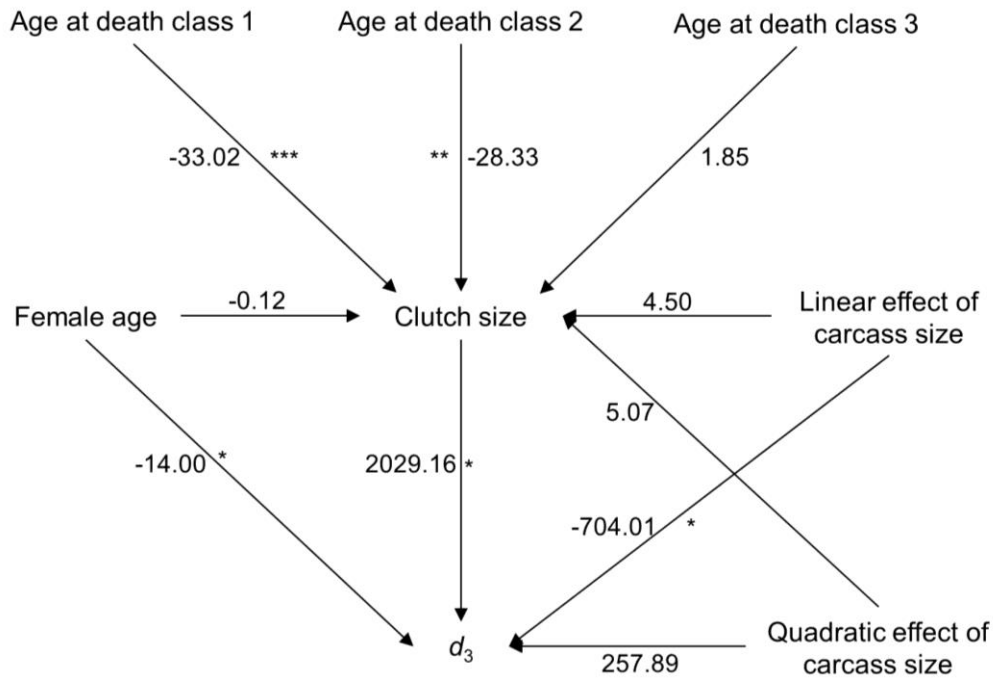



Figure 5.3: Path diagram showing the direct and indirect effects of age on $d_3$. Asterisks indicate significance (* indicates P < 0.05, ** indicates P ≤ 0.01, *** indicates P ≤ 0.001). The linear and quadratic effects of carcass size are defined as orthogonal and therefore no correlation between them is represented.

### 5.3.3 *Combined effects of age on laying metrics*

The total effects of age on the laying metrics follow from the combined direct and indirect effects. As a result of the direct effects of age and the indirect effects mediated though clutch size, the total effect of female age results in an increase in $d_1$ of 2.69h, a decrease of 5.46h$^2$ in $d_2$ and a decrease of 249.62h$^3$ in $d_3$ for each additional day of female age. Older females laid earlier and produced clutches with a smaller variance in laying times and a less positively skewed distribution of laying times (Figure 5.4).

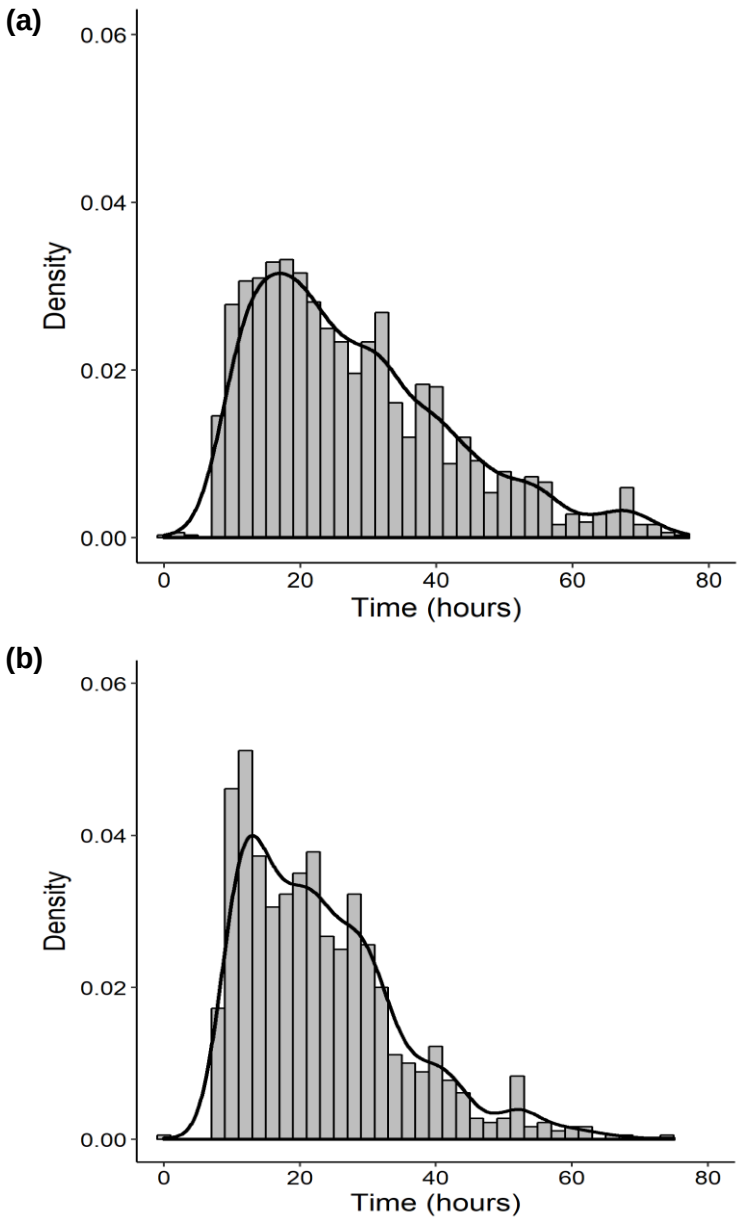



Figure 5.4: Density histogram of eggs laid in hours after the female was placed on the carcass for (a) young females aged 15-35 days post-eclosion and (b) old females aged 55-77 days post-eclosion.

## 5.4 DISCUSSION

I investigated the effect of female age on higher moments of within-brood distributions of hatching times in *N. vespilloides*, using laying times as a proxy for hatching times because they can be measured more accurately than hatching times (Smiseth *et al*., 2006). Ageing caused older females to delay laying and to produce clutches with less variance and less strongly positive-skewed laying times. Although I lack the requisite formal evolutionary models to make informed evolutionary inferences about how I might expect senescence to manifest on higher central moments of within-brood distributions, I can use theory and experimental data from behavioural ecology to suggest the direction of selection on the laying metrics. From this, I propose a verbal argument for the expected changes in laying times with age that would result from weakening selection (Medawar, 1952, Williams, 1957, Hamilton, 1966). Below I discuss the implications of my results in light of expectations for how higher moments of hatching time distributions might affect offspring growth and survival.

Directional selection for early laying or hatching times is predicted under many circumstances by verbal arguments and theoretical models (Clark and Wilson 1981). For example, the hurry-up hypothesis predicts directional selection for an earlier mean hatching time when breeding conditions decline over time (Hussell, 1972; Clark and Wilson, 1981; Slagsvold, 1986). There is also evidence from *N. vespilloides* that delaying laying for too long after encountering the carcass is unfavourable because microbial growth increases over time with a detrimental impact on egg survival (Jacobs *et al*., 2014) and larval growth (Rozen *et al*., 2008). However, there is some limit to how soon females can lay because they do not mature their oocytes until they secure a carcass for breeding (Wilson and Knollenberg 1984). Therefore, laying eggs too soon after encountering the carcass may be costly if females have not had sufficient time to feed from the carcass to mature their oocytes, resulting in eggs laid too early being of poorer quality. This constraint is only likely to affect laying within a few hours of encountering the carcass. Over likely ranges of laying times, I might expect directional selection for a reduced time until laying. If the strength of selection is reduced with age, as predicted by classical ageing theory (Medawar, 1952; Williams, 1957; Hamilton, 1966), I therefore expect an increase in the time until eggs are laid as female age at breeding increases. My results support this prediction.

There is evidence from previous *N. vespilloides* experiments that offspring survival is greatest when the time between the first and last larvae hatching (termed spread) is 12h (Smiseth *et al*., 2008). Larval survival declined when the larvae were placed on the carcass simultaneously (Smiseth *et al*., 2008) or over more than 12h, with greater mortality and lower average larval mass when the larvae hatched over 48h compared to 24h (Smiseth and Morgan, 2009; Ford and Smiseth, 2016). These experiments manipulated the spread rather than the variance in within-brood hatching times *per se*, and the hatching patterns were artificial. However, in the present study the laying spread and within-brood variance are very highly correlated (Pearsons' correlation: $r = 0.78$, $t_{133} = 14.49$, $P < 0.0001$) so the relationship between offspring survival and within-brood variance is likely to be similar to the relationship with spread. If females of prime age produce clutches with a within-brood variance close to the optimum, I might expect increasing maternal age to be associated with greater scatter around the optimum in the absence of mutational bias (Charlesworth, 1990). However, in the present study the average laying spread for females of prime reproductive age early in life (11-35 days post-eclosion), when the strength of selection for most traits can be assumed to be greatest, was 40h and none of these females produced a clutch with a spread less than 12h. This suggests that over the biologically relevant range there is likely to be directional selection for smaller within-brood variance. In line with this, a previous *N. vespilloides* study found that offspring survival decreased with increasing laying spread (Ford and Smiseth, 2017). I might therefore expect the within-brood variance to increase with increasing maternal age as selection gets weaker (Medawar, 1952, Williams, 1957, Hamilton, 1966). However, I found the opposite: older females produced clutches with less variance in laying times.

The skew in hatching times is important when offspring interact with each other after hatching because it influences the competitive asymmetries between siblings (Mock, 1984; Magrath, 1990; Stoleson and Beissinger, 1995; Mock and Parker, 1997; Parker *et al*., 1989). The brood reduction hypothesis (Lack, 1947) and the insurance hypothesis (Stinson, 1979) propose that asynchronous hatching leads to the production of "core" offspring that hatch first and "marginal" offspring that hatch later (Forbes, 1990). The marginal offspring may serve as insurance against mortality of the core offspring (e.g. the insurance hypothesis, Stinson, 1979), and these generally survive only if the core offspring fail to hatch or die soon after hatching (Mock and Parker, 1986). Alternatively, the production of marginal offspring

may allow parents to adjust brood size to match resource availability with minimal waste of parental time and effort (Forbes, 1990) by acting as expendable offspring that can survive when resources are plentiful but not when resources are scarce (brood reduction hypothesis, Lack, 1947). There is evidence from other *Nicrophorus* studies that later-hatched marginal offspring have lower growth rates and higher mortality rates than core offspring (Smiseth *et al*., 2007a; Takata *et al*., 2013). The number of offspring surviving to independence is likely to be maximised when the distribution of hatching times is positively skewed because this produces a relatively smaller number of marginal offspring compared to core offspring, and indeed, very few females (8%) produced clutches with negative skews. However, if hatching times are too strongly positively skewed, then the last offspring to hatch will almost certainly die and therefore represent a waste of female resources which otherwise could have been invested into the remaining offspring. This suggests that over the range of skews females actually produce there is likely to be directional selection for less strongly positive skews. Evidence in support of this was found in a previous *N. vespilloides* study where larval survival was lower in broods with more strongly positively skewed distributions of hatching times (Ford *et al*., 2018). If the strength of selection is reduced with age (Medawar, 1952; Williams, 1957; Hamilton, 1966), I might therefore expect older females to produce clutches with more strongly positively skewed distributions of laying times. However, I found that skew became less positive with increasing female age.

The total effect of age on the laying metrics results from a combination of direct effect of female age and the indirect effect of female age mediated through its effects on clutch size. This indirect effect was far more important than the direct effect of maternal age for all laying metrics. Reproductive senescence is predicted by evolutionary theory (Hamilton, 1966; Charlesworth, 1994, 2001), and it is common in many taxa (reviewed in Nussey *et al*., 2013). Clutch size is an important fitness trait, and reproductive senescence is often manifested as a decrease in clutch size with age (Begon and Parker, 1986). For example, the number of eggs laid declines when females breed at older ages in many studies of senescence in *Drosophila* (Miller *et al*., 2014), several species of fish (Patnaik *et al*. 1994) and birds (Martin, 1995; Reid *et al*., 2003b; McCleery *et al*., 2008). Clutch size can influence many other traits, including the distributions of laying times (Smiseth *et al*., 2008; Botterill-James *et al*., 2017). I found a trend towards smaller clutches as female age increased, with evidence for selective disappearance of those females that

produced fewer eggs. A similar pattern has also been observed in birds (Reid *et al*., 2003b; McCleery *et al*., 2008). These results demonstrate the importance of accounting for the potential for selective disappearance of poorer quality individuals in studies of ageing (Reid *et al*. 2003b; van de Pol and Verhulst, 2006; McCleery *et al*., 2008; Blas *et al*., 2009; Bouwhuis *et al*., 2009; Hayward *et al*., 2013).

My results also showed that changes in the distributions of laying times were directly affected by female age over and above the effects of clutch size; all laying metrics decreased as female age increased. I can exclude any effects of previous breeding experience or physiological wear through repeated breeding attempts that might confound the effects of age in longitudinal studies because all females bred only once. Additionally, there was no evidence for differential mortality of females directly shaping the distributions of laying times because age at death had no statistically significant effect on any laying metric (although it did affect clutch size). Although the changes I observed in variance and skew with increasing maternal age initially appear contrary to expectations under senescence, there are a number of reasons why selection may not act on within-brood distributions of hatching times as I expected. Firstly, a given laying metric could have opposite effects on different measures of fitness. For example, greater skew is likely to be associated with increased numbers of offspring but reduced offspring survival. Selection for greater egg production could result in indirect selection for a greater skew while selection for increased offspring fitness could cause indirect selection for reduced skew. This makes it difficult to estimate the direction of total selection on each laying metric. Secondly there could be negative phenotypic correlations between laying metrics and other fitness-related traits (Price and Langen, 1992), which could constrain hatching patterns. There may be trade-offs between laying metrics and other reproductive traits or prospects of future reproduction (Stearns, 1989), and these trade-offs may also change with age. Thirdly, a trait such as skew in hatching times can appear to be under selection if an environmental variable affects both the trait and the measure of fitness (Price *et al*., 1988). For example, a variable such as carcass quality could potentially produce a negative correlation between skew and offspring survival if feeding on a high quality carcass aids female oocyte maturation (Trumbo and Robinson, 2004). This could lead to a less strongly skewed distribution of laying times and increased larval survival through improved nutrition. The changes in within-brood variance and skew with age may be consistent with senescence if the total selection on these laying metrics is positive.

My expectations regarding adaptive hatching patterns are not based on formal models and can only be extended to evolutionary predictions if linear and higher-order selection gradients are estimated in beetles of prime age. There are a number of difficulties in obtaining this information about hatching patterns in *N. vespilloides*. To measure the effect of distributions of laying times within a clutch on offspring fitness in such a way as to decouple these effects statistically from those arising from individual laying times, it would be necessary to follow each larva until it eclosed as an adult and reproduced, recording the timing of laying of the egg it hatched from (or the time when it hatched) and the relative laying (or hatching) times of all other offspring in the brood. However, there is no unobtrusive way to uniquely mark eggs or larvae without physically separating them, and it would be undesirable to do so because the effect of hatching time on fitness is partly mediated by interactions between siblings. Furthermore, it is likely that many reproductive traits in *N. vespilloides* are not independent of each other, and many phenotypic traits must also be measured in order to disentangle indirect selection through correlated traits (Lande and Arnold, 1983). Estimation of selection gradients may be more feasible for other maternally-controlled traits that introduce variation in offspring fitness within a clutch or litter, such as egg size (McGinley *et al*., 1987; Koops *et al*., 2003), size at hatching or birth (Marshall *et al*., 2002, 2008), allocation of hormones or nutrients to eggs (Muller and Groothuis, 2013; Leal *et al*., 2013), and allocation of resources to young after hatching or birth (Gilby *et al*., 2011). The effect on fitness of within-clutch variation in traits such as egg size may be more easily elucidated (e.g. Williams *et al*., 1993; Williams, 1994; Viñuela, 1997; Kudo, 2001; Hubner *et al*., 2002; Bosman, 2014), but information on how within-clutch distributions of these traits change with age is lacking. I urge future studies to investigate how senescence affects higher moments of traits under maternal control to further our understanding of the evolution of ageing.

# Chapter 6

# General Discussion

In this thesis, I have explored the causes and consequences of asynchronous hatching in *N. vespilloides*. I measured hatching asynchrony in terms of laying spread and laying skew, except in Chapter 5, where I treated the timing of laying of each egg as a separate observation. Asynchronous hatching may be adaptive or may be the result of constraints on laying. I investigated a potential adaptive reason for the occurrence of asynchronous hatching (sexual conflict over parental care in Chapter 2), and potential nonadaptive causes due to physiological constraints and female condition (hurry-up hypothesis in Chapter 3, inbreeding status in Chapter 4 and age in Chapter 5). Hatching asynchrony can be influenced by both intrinsic and extrinsic factors. Intrinsic factors include aspects of the female's state, such as her age, body size or inbreeding status. Extrinsic factors include environmental conditions such as carcass availability, or can be driven by interactions with congeners, including competition and sexual conflict. These factors can influence hatching asynchrony directly or indirectly, for example, through their effects on clutch size. In this thesis I explored both intrinsic (female inbreeding status in Chapter 4 and female age in Chapter 5) and extrinsic factors (sexual conflict over parental care in Chapter 2, breeding resource quality and quantity in Chapter 3) that could cause asynchronous hatching. I assessed the consequences of asynchronous hatching in terms of offspring performance and cost to females.

### 6.1 *General findings*

In all experiments, I found that laying skew index was generally negative, indicating most eggs were laid in the first half of the laying period (mean -0.20, range -0.76-0.46). Across all experiments, females took on average 17.24 hours to start laying after being placed on the carcass (range 2-98h). They laid 35.81 eggs on average (range 5-83) and took 36.14 hours from the first to last egg (laying spread) on average (range 4-124h). Different aspects of laying pattern were affected independently; carcass decomposition and clutch size affected laying spread, but had no significant effect on laying skew, while maternal inbreeding affected laying skew but not laying spread. This is in accordance with the findings of Smiseth *et al*. (2008) and Takata *et al*. (2013, *N. quadripunctatus*), who found no correlation between hatching spread and hatching skew. My results indicate that laying asynchrony is plastic in *N. vespilloides* and that females may facultatively adjust their laying patterns in response to breeding conditions (Chapter 3) and their own state (Chapter 4, Chapter 5).

### 6.2 *Consequences of asynchronous hatching for offspring*

I found that larval survival was reduced when laying was more strongly positively skewed towards the start of the laying period (Chapter 4), probably because the last-hatched offspring have higher mortality when there is a greater disparity in age and therefore size between them and the first-hatched larvae. In the carcass decomposition experiment, I found that the absolute number of larvae and the proportion of the eggs that hatched and survived to dispersal as larvae was lower in broods with a greater hatching spread (Chapter 3). Additionally, I found that experimentally generated hatching asynchrony reduced the number of larvae surviving from hatching to dispersal and decreased larval mass at dispersal compared to all larvae being placed on the carcass simultaneously (Chapter 2). Larval mass is an ecologically relevant measure of the fitness consequences of asynchronous hatching to offspring in this species because smaller larvae become smaller adults (Bartlett and Ashworth, 1988), which in turn are less successful in competition for carcasses (Otronen, 1988; Scott and Traniello, 1990; Trumbo, 1991; Robertson, 1993), lay fewer and smaller eggs (Steiger, 2013), and have shorter lifespans (data from Chapter 5, Pearson's correlation $t_{126} = 2.49$, $P = 0.014$, $r = 0.216$). Larger females are capable of laying a greater number of eggs per hour (Steiger, 2013), and therefore could lay a clutch of a given size more synchronously, thereby increasing the fitness of their offspring. Thus, the degree of hatching asynchrony of a clutch could affect the laying patterns produced by females hatching from that clutch when they themselves breed.

### 6.3 *Consequences for females*

Given that hatching asynchrony reduces offspring fitness, it is surprising that females do not always lay their eggs as synchronously as possible. There is no evidence of a cost to females of caring for synchronous broods compared to asynchronous broods (experimentally generated hatching spreads in Chapter 2; Smiseth and Morgan, 2009). Currently there is no information on the cost of caring for broods with differing hatching skews. There is a potential benefit of producing a greater clutch size, which is associated with greater laying spread, rather than of producing a clutch with a greater laying asynchrony *per se* (see below). However, females appear to be capable of laying a given clutch size more synchronously in some circumstances. For example, I found that females laid the same number of eggs regardless of carcass decomposition but laid much more synchronously on

decomposed carcasses. This suggests that females could gain the benefits of laying a greater number of eggs without also increasing laying asynchrony. However, there may be a cost to females of producing clutches with smaller laying spreads and skews because laying several eggs in rapid succession may be physiologically demanding. If so, producing clutches with smaller laying spreads and skews should reduce female lifespan or her mass gain while breeding. Contrary to this expectation, I found that females that started to lay sooner and produced clutches with a smaller laying spread lived longer (Addendum to Chapter 2), suggesting that there is not a cost manifested as reduced lifespan. However, it is not possible to experimentally induce females to produce clutches with a particular laying pattern, only to correlate laying spread or skew with female lifespan. As a result, the effect of producing a certain laying pattern on lifespan is confounded with the effect of factors that influence both laying pattern and lifespan. For example higher quality females may be able to start laying sooner, lay more synchronously and live longer.

### 6.4 *Clutch size*

Clutch size can potentially both cause, and be constrained by, asynchronous hatching. I found that greater laying spread was associated with larger clutches (Chapter 2, carcass size experiment Chapter 3), which was also found by Smiseth *et al*. (2008) and Botterill-James *et al*. (2017). However this is not always the case (e.g. Smiseth *et al*., 2006; Takata *et al*., 2013 *N. quadripunctatus*); I found that females producing inbred offspring laid a smaller number of eggs but this was not accompanied by a significant reduction in laying spread (Chapter 4), and females breeding on decomposed carcasses produce the same number of eggs with a smaller laying spread than females breeding on fresh carcasses (Chapter 3). In cases where laying synchrony is constrained by clutch size, a greater level of hatching asynchrony may allow for the production of a larger clutch size because more eggs are laid if females continue to lay for longer. The total clutch size is increased by around 8 eggs for clutches with a laying spread of 48h compared to 24h (calculation from linear regression equation, data from Chapter 2) whereas on average 4 fewer larvae out of the initial 20 larvae survived to dispersal in highly asynchronous broods (48 hour hatching spread) than in moderately asynchronous broods (24 hour hatching spread) (data from Chapter 2). If the additional eggs hatch and survive, this positive effect of hatching asynchrony on larval number could potentially (at least partially) counteract at least some of the negative effect of highly

asynchronous hatching on offspring fitness. Conversely, the rate of laying may limit clutch size (Steiger, 2013) because if larger clutches take longer to lay, very large clutches would have a long hatching spread, which would have a detrimental effect on larval survival, negating the benefit of producing the additional eggs. I found no evidence that the greater laying spread concomitant with increasing clutch size as carcass size increases is detrimental to larval survival (carcass size experiment Chapter 3). This might reflect that, on large carcasses, the additional food supply may be sufficient to rear a larger, more asynchronous brood without compromising offspring size or survival. Further work is needed to determine whether greater clutch size associated with asynchrony is sufficient to provide a net benefit of asynchronous hatching to females, and how this depends on factors such as carcass size.

### 6.5 *Sexual conflict hypothesis (Chapter 2)*

The sexual conflict hypothesis for the evolution of asynchronous hatching suggests that females adjust hatching patterns in order to increase male parental effort relative to female effort. I found that females could manipulate males to remain with the brood longer by laying more asynchronously, but this came at a substantial cost as it reduced larval survival and mass. I also found that males responded to earlier female desertion by remaining with the brood for longer. However, I did not find a benefit of increased male residency time because female mass change and lifespan were unaffected by male assistance. This suggests that manipulating the male through hatching patterns is not worthwhile and, indeed, I found no evidence that females attempted to do so because their laying patterns did not differ when breeding in the presence or absence of a male. However, when I repeated the experiment allowing pairs to mate before encountering a carcass, females did produce clutches with a greater laying spread when they were accompanied by the male on the carcass, but this was probably due to them starting to lay earlier (Addendum to Chapter 2). The intensity of sexual conflict over parental care may vary depending on the context (see below). There may be reduced conflict when there is a lower chance of future reproduction and the current reproductive attempt is particularly valuable (Ward *et al*., 2009), such as when there is particularly intense competition for carcasses, or when the parents are old or small. When sexual conflict over parental care is intense and male assistance is particularly valuable to the female it may be worthwhile for females to manipulate males to remain with the

brood longer by laying asynchronously despite the cost associated with asynchronous hatching, but this was likely not the case in these experimental conditions.

### 6.6 *Hurry-up hypothesis (Chapter 3)*

The hurry-up hypothesis suggests that completing reproduction as soon as possible is favoured when the quantity or quality of resources used for breeding declines over time, leading to hatching asynchrony as a by-product. I conducted two experiments in which I provided females with an incentive to complete reproduction sooner by giving them carcasses that varied either with respect to size (resource quantity) or decomposition (resource quality). In line with the predictions of the hurry-up hypothesis, I found that larvae dispersed from the carcass earlier when females commenced oviposition sooner and that laying spread was greater when females commenced egg laying earlier. However, I found no evidence that females commenced egg laying earlier on either decomposed or larger carcasses. Unexpectedly, females laid the same number of eggs, but with a smaller laying spread, when breeding on decomposed carcasses compared to fresh carcasses. Females produced clutches with a greater laying spread on larger carcasses, but only because clutch size increased with carcass size and females took longer to complete larger clutches. Therefore my results are inconsistent with the hurry-up hypothesis.

### 6.7 *Inbreeding (Chapter 4)*

I expected that inbred females might produce different laying patterns to outbred females if they are of poorer quality and this constrains laying. I found that inbred females produce clutches with a different laying skew to outbred females. However, egg laying was less strongly skewed towards the early part of the laying period in clutches produced by inbred mothers compared to outbred mothers. This reduction in the skew in egg laying is beneficial for larval survival, suggesting that inbred females adjusted their laying patterns facultatively, thereby partially compensating for the detrimental effects of maternal inbreeding on offspring hatching success and survival. In contrast, I found that females did not adjust their laying patterns (laying spread or laying skew) in response to breeding with a relative and thereby producing inbred offspring.

### 6.8 *Female age (Chapter 5)*

In this chapter I looked at changes in laying patterns with age, using each egg as an observation. I used three laying metrics, $d_1$, $d_2$, and $d_3$. Clutch-specific averages of these measures are the within-clutch means, variances, and skews, respectively, for the timing of egg laying. Females breeding at older ages laid later and produced clutches that had a smaller variance in laying times and were less positively skewed (laying was less skewed towards the start of the laying period). There was a trend towards decreased clutch size with age and this was associated with later laying, reduced within-clutch variance in laying times and a less positively skewed distribution of laying times. The changes in laying pattern with age were not solely the result of decreasing clutch size.

### 6.9 *Compensation*

I expected that both increased age at breeding and level of inbreeding would have similar effects on laying patterns, because they both potentially represent poorer female 'quality'. Consequently, I predicted that older and more severely inbred females would be constrained to produce clutches with suboptimal laying patterns (greater laying spread and more extreme laying skew). However I found evidence that the opposite was the case for both age and inbreeding status; older females and highly inbred females produced clutches with laying patterns that were more beneficial to offspring. This suggests that females may facultatively adjust their laying patterns in response to their state, thereby potentially compensating for being constrained in providing other aspects of parental care. As well as responding to their own state, females may also compensate for environmental conditions that are inimical to offspring growth and survival by laying more synchronously. In support of this, I found that females responded to the state of decomposition of the carcass by laying more synchronously on decomposed carcasses compared to fresh carcasses. In the case of carcass decomposition, laying more synchronously has the additional benefit of laying being completed in a shorter period of time, allowing eggs to hatch and larvae to develop during the earlier stages of microbial colonisation. Adjusting laying patterns may be more effective in improving offspring growth and survival than devoting more resources to other aspects of breeding, such as increasing egg size or number of eggs, because hatching patterns can have a greater effect on offspring performance than other maternally-controlled traits (Bitton *et al*., 2006). Additionally, increasing egg size, egg number, or post-hatching

care may be more costly than adjusting laying patterns and might not possible due to physiological constraints associated with female state.

Female quality is likely to decline with age as the strength of selection for survival and reproduction gets weaker (Medawar, 1952; Williams, 1957; Hamilton, 1966). For instance, I found that older females lay smaller eggs ($t_{133}$= -2.24, $P$ = 0.027, $r$ = -0.191, Pearson's correlation, egg size calculated as the product of length and width of the first egg laid in each clutch that was suitable for measurement on ImageJ), which is detrimental to offspring performance because egg size is positively correlated with larval mass (Monteith *et al*., 2012). Lock *et al*. (2007) also found that initial offspring size was affected by female age and that this was compensated for by differences in the amount of post-hatching parental care provided by females of different ages. As a result, offspring size at dispersal was the same whether they were produced and cared for by females aged 2 weeks or 6 weeks post-eclosion. Given that the effect of egg size on offspring performance is masked by the more benign conditions generated by the presence of parental care after hatching (Monteith *et al*., 2012), it is reasonable to expect it may also be overridden by the more benign post-hatching environment generated by the reduced variance and skew in laying times that I found to occur with increasing female age (Chapter 5). Rather than resulting from senescence, the reduction in the degree of hatching asynchrony with age may represent another mechanism for compensating for declines in reproductive traits with age.

Females may also be of poorer quality when they are highly inbred, which can have detrimental effects on offspring fitness (Mattey *et al*., 2013; Chapter 4). I found that laying skew index was less negative for clutches produced by inbred females compared to outbred females, which shows that the timing of laying was less positively skewed towards the beginning of the laying period for inbred females. Larval survival is increased when laying times are less strongly positively skewed (Chapter 4). Early-hatched offspring are competitively superior to offspring that hatch later (Lack, 1947; Stinson, 1979) and these competitive asymmetries between offspring are greater when laying times are more strongly skewed (Magrath, 1990; Stoleson & Beissinger, 1995; Mock & Parker, 1997). Late-hatched larvae have reduced growth rates and are less likely to survive to independence than early-hatched larvae (Smiseth *et al*., 2007a; Takata *et al*., 2013). A greater skew in laying times is more likely to result in mortality of late-hatched larvae because they are increasingly competitively disadvantaged. Therefore, my results suggest that

females facultatively compensate for the detrimental effects on their offspring of having an inbred mother by reducing their laying skew. Conversely, I found no evidence that females altered their laying pattern (laying spread or laying skew) in response to producing inbred offspring as a result of mating with a relative. Females may be able to compensate for offspring inbreeding through increased allocation of hormones, nutrients or other components to eggs (Ihle *et al*., 2017), or by providing a greater quantity or quality of post-hatching care (Mattey *et al*., 2018), rather than through greater laying synchrony.

### 6.10 *Sibling competition*

The effects of asynchronous hatching on offspring are mediated through asymmetric sibling competition (Mock, 1984; Magrath, 1990; Stoleson and Beissinger, 1995; Mock and Parker, 1997; Parker *et al*., 1989). Larvae develop together on the carcass and therefore interact after hatching, raising the potential for competition between siblings. The carcass is a limited resource providing the sole source of food for the larvae during development. The larvae compete to gain access to the parents' mouthparts to receive pre-digested carrion (Smiseth *et al*., 2003), which increases their growth compared to self-feeding (Lock *et al*., 2004). The last offspring to hatch are competitively disadvantaged because they are much smaller than their siblings. Larvae undergo a seven-fold increase in body mass during the first 24 hours after hatching (Smiseth *et al*., 2003) so, in a brood with an average laying span, by the time the last larvae hatch the first-hatched larvae will be more than seven times their size. Early-hatched larvae are more successful at accessing the parents' mouthparts (Andrews and Smiseth, 2013) and grow more quickly (Smiseth *et al*., 2007a). Greater hatching asynchrony increases the disparity in competitive abilities of the larvae.

Sibling competition is affected by both brood size and resource availability (carcass size) (Sieber *et al.*, 2017; Botterill-James *et al*., 2017), which also have indirect associations with asymmetric sibling competition through asynchronous hatching. Brood size is limited by clutch size, which in turn is influenced by carcass size (Chapter 3, Botterill-James *et al*. 2017). Hatching is more asynchronous when clutch sizes are larger (Chapter 3, Smiseth *et al*. 2008; Botterill-James *et al*. 2017) and greater hatching asynchrony exacerbates competitive asymmetries between siblings (Mock, 1984; Magrath, 1990). Thus, some of the factors influencing asynchronous hatching may also affect sibling competition directly, as well as

indirectly through their effect on laying patterns. Competition may be more intense on smaller carcasses compared to larger carcasses, but also less asymmetric if smaller clutch sizes laid on smaller carcasses allow greater laying synchrony because the competitive abilities of the larvae are more similar. Conversely, larvae in larger broods may experience both more intense competition (Godfray and Parker, 1992) and more asymmetric competition due to the greater laying asynchrony associated with larger clutches. Sibling competition reduces the optimum clutch size parents should produce (Godfray and Parker, 1991, 1992). However, clutch size could in turn affect sibling competition through its effect on hatching asynchrony. Additionally, females may have some influence over the amount and asymmetry of competition between their offspring through facultative control over their hatching patterns, which may contribute to the outcome of parent-offspring conflict over resource division between larvae in a brood.

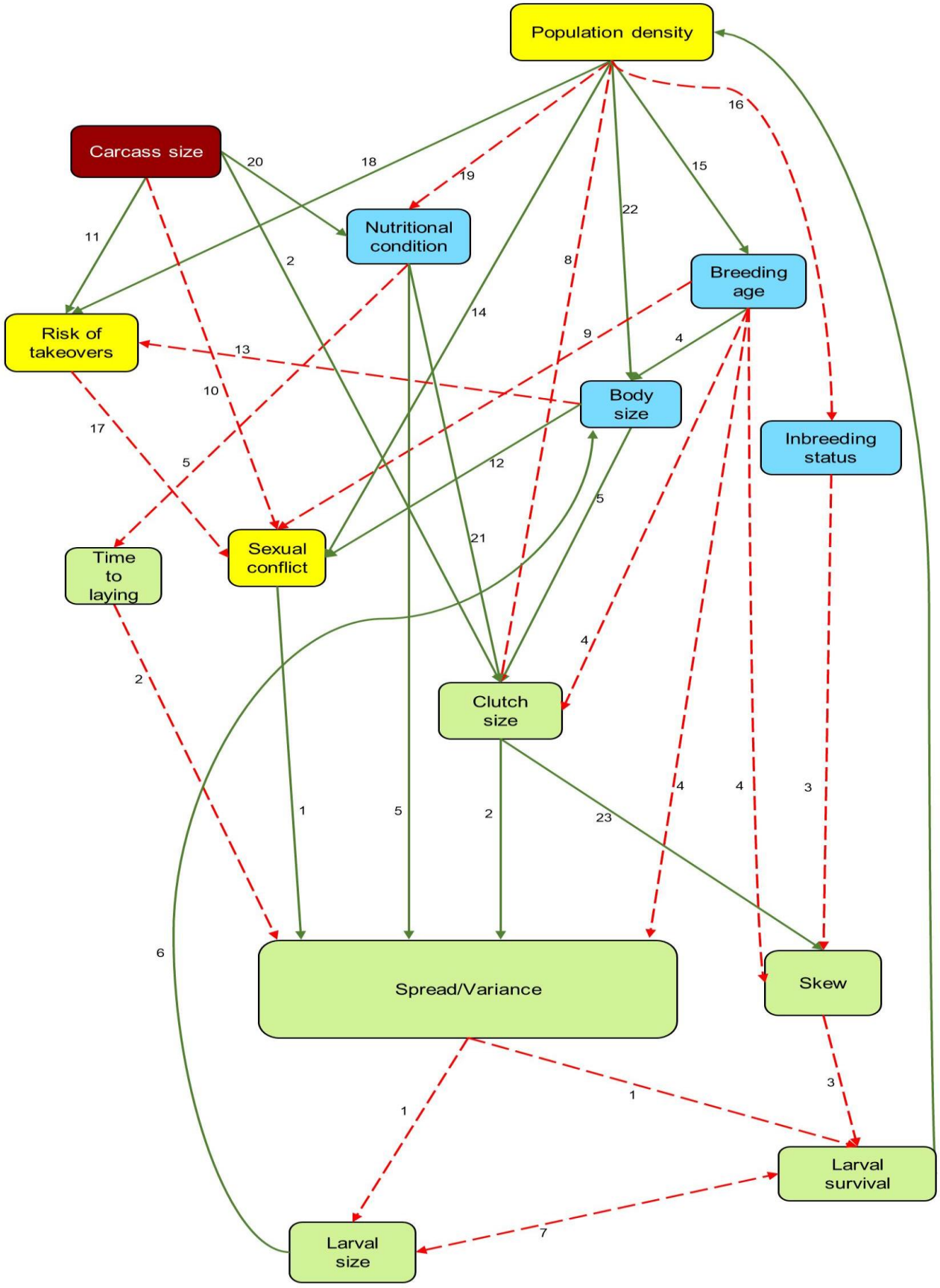
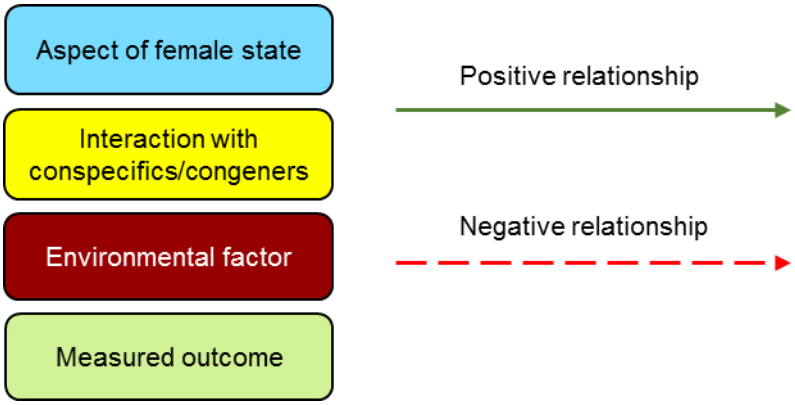



Figure 6.1: Causes and consequences of asynchronous hatching and their interactions.

Key:




Numbers on arrows represent chapters in this thesis or previous studies that provide evidence for the links depicted:

1. Chapter 2
2. Chapter 3
3. Chapter 4
4. Chapter 5
5. Steiger, 2013
6. Bartlett and Ashworth, 1988
7. Bartlett, 1987; Bartlett and Ashworth, 1988; Smiseth *et al*., 2014
8. Creighton, 2005 (*N. orbicollis*); Rauter *et al*., 2010 (*N. pustulatus*)
9. Adler and Bonduriansky, 2014
10. Bartlett, 1988; Kishida and Suzuki, 2010 (*N. quadripunctatus*)
11. Trumbo, 1991
12. Smith *et al*., 2014; Pilakouta *et al*., 2015b
13. Otronen, 1988
14. Scott, 1998a (*N. orbicollis*); Hopwood *et al*., 2015; Pilakouta *et al*., 2016b
15. Newton, 1992; Ferrer *et al*., 2004; Trinkel *et al*., 2010
16. Keller and Waller, 2002
17. Trumbo, 2006
18. Trumbo, 1990a
19. Steiger *et al*., 2007; Trumbo and Robinson, 2004
20. Wilson and Knollenberg, 1984
21. Trumbo and Robinson, 2004; Steiger *et al*., 2007; Steiger, 2013
22. Creighton, 2005 (*N. orbicollis*)
23. Smiseth *et al*., 2008

### 6.11 *Causes and consequences of asynchronous hatching*

In light of my findings, and the existing literature on asynchronous hatching in burying beetles and birds, I have compiled a diagram showing the main causes and consequences of asynchronous hatching in *N. vespilloides* (Figure 6.1). Asynchronous hatching can be caused by extrinsic (e.g. environmental factors or intraspecific interactions) or intrinsic factors (e.g. aspects of female state). These can influence hatching patterns directly, or indirectly through their effects on clutch size or the delay until laying after the female encounters the carcass. The resulting hatching asynchrony may be adaptive or the nonadaptive by-product of constraints on laying.

An extrinsic factor that could lead to adaptive asynchronous hatching is sexual conflict over parental care. On very small carcasses the female may benefit from the male deserting because there is insufficient food for both parents and the larvae (Bartlett, 1988) but on most carcasses male assistance in parental care can be beneficial. Sexual conflict over the duration of care provided by each parent could then arise when each parent attempts to shift more of the work onto their partner, allowing them to reduce their own contribution. Females may attempt to manipulate males to increase their duration of care by laying more asynchronously under circumstances where the benefit of a greater duration of male care outweighs the detrimental effects of hatching asynchrony on the offspring (Chapter 2).

The intensity of sexual conflict could be affected by other intraspecific interactions such as competition, and environmental factors which affect competition, specifically carcass size (Bartlett, 1988; Kishida and Suzuki, 2010). Carcasses are a scarce and valuable breeding resource (Trumbo, 1990a). Competition for carcasses is intense and beetles that have not secured their own carcass will attempt to take over carcasses that are already being utilised. If the carcass is successfully usurped, the intruders will kill any larvae that are already present (Trumbo, 1990a). Larger carcasses are more valuable because they can support a greater number of larvae, and therefore attempts at usurpation are more likely (Trumbo, 1991). A higher risk that the carcass will be usurped by conspecifics or other *Nicrophorus* species may incline the resident parents to cooperate to prevent a takeover of the carcass, because both parents can defend the carcass more effectively than a single female (*N. orbicollis*, Trumbo, 2006; *N. quadripunctatus*, Suzuki, 2011). In support of this, male residency time is greater on larger carcasses (Bartlett, 1988). Competition for carcasses increases with

population density (Trumbo, 1990a). Males increase their residency time in response to cues that population density is high (*N. orbicollis*, Scott, 1998a) or that there is greater reproductive competition (Hopwood *et al*., 2016), and females provide a greater amount of care if they have experienced a contest (Pilakouta *et al*., 2016b), which is more likely at higher densities. Sexual conflict over parental care is likely to be more intense between parents breeding on smaller carcasses and at low population densities, when the risk of takeovers is reduced. In these circumstances, the resolution of this sexual conflict over parental care could be mediated by asynchronous hatching because males remain longer with more asynchronous broods (Chapter 2).

Sexual conflict can also be influenced by intrinsic factors pertaining to the parents' state. For example, whether females attempt to manipulate males to remain with the brood longer through asynchronous laying may depend on their body size, because sexual conflict over parental care is likely to be more intense when the parents are larger. The reason for this is that larger males desert the brood sooner (*N. orbicollis*, Smith *et al.*, 2014; *N. vespilloides*, Pilakouta *et al*., 2015b), potentially increasing sexual conflict over parental care if females are forced to increase their contribution. Additionally, larger beetles are better able to defend their brood against intruders (Otronen, 1988), potentially allowing one parent to desert the brood early without risking brood failure if their partner is large enough to defend the brood alone. When both parents are small, cooperation may be necessary to meet the demands of the larvae. In support of this, small females provided more care when paired with a small male (Pilakouta *et al*., 2015b). Smaller females may also have shorter lifespans and therefore have less incentive to desert the brood because they may not survive long enough to have another breeding attempt. Similarly, the intensity of conflict over parental care may also decrease with age (Adler and Bonduriansky, 2014) if parents are willing to invest more in current reproduction because there is a reduced cost of missing the dwindling opportunities for future breeding attempts. Aspects of the parents' state such as age and body size could therefore influence the intensity of sexual conflict over parental care, determining whether the female attempts to manipulate the male through greater hatching asynchrony.

Female state can also affect hatching asynchrony directly. In Chapter 5, I found a direct effect of age on laying asynchrony, with older females producing more synchronous clutches with a lower variance and less positive skew in laying times. I

also found evidence of selective disappearance of females producing small clutches (Chapter 5). When population density is high, the average breeding age of females may increase (e.g. Newton, 1992; Ferrer *et al*., 2004; Trinkel *et al*., 2010) because it takes longer to find a suitable carcass for breeding when there is intense competition over carcasses. I found that females surviving to breed at older ages were larger (data from Chapter 5). Larger beetles are better able to compete for carcasses (Otronen, 1988) and the average size of breeding beetles is greater at high densities (*N. orbicollis*, Creighton, 2005). Furthermore, larger females are able to produce larger clutches (Bartlett and Ashworth, 1988; Steiger, 2013), which could explain why I did not see a significant decrease in clutch size with age once selective disappearance was accounted for. Inbreeding is another aspect of female state that affects laying patterns. Low population density may increase the likelihood of inbreeding due to the scarcity of suitable mates (Keller and Waller, 2002). In Chapter 4, I found that inbred mothers produce clutches where laying is less strongly positively skewed towards the start of the laying period. Larval survival is increased when laying times are less positively skewed (Chapter 4). By producing clutches with less skewed laying times, inbred females could facultatively compensate for the detrimental effects on their offspring of having an inbred mother.

Many factors affect laying patterns indirectly through their effects on clutch size or the delay until laying. One such factor is the intensity of competition over carcasses, which is affected by carcass availability and population density. Interspecific competition for breeding opportunities provides an incentive to complete reproduction quicker (Taylor and Perrin, 2008), which could increase hatching asynchrony because laying sooner after encountering the carcass is associated with a greater laying spread (Chapter 3). Females may begin to lay as soon as possible upon encountering a suitable carcass when competition is intense because the value of the carcass declines rapidly once the resident larvae have begun to consume it, reducing the risk of takeovers (Trumbo, 2006). Larger clutches are also associated with a greater laying spread (Chapter 3, Smiseth *et al*. 2008; Botterill-James *et al*., 2017). Although the limited breeding hypothesis (Beissinger and Waltman, 1991) predicts that females should produce larger clutch sizes when there is more competition, brood size is actually smaller on a given carcass size at higher densities (*N. orbicollis*, Creighton, 2005; *N. pustulatus*, Rauter *et al*., 2010). This could be explained by the fact that there is a trade-off between the size and number of larvae (Chapter 3, Bartlett, 1987; Bartlett and Ashworth, 1988; Smiseth *et*

*al*., 2014). At low population densities, it may be preferable for females to produce a large number of larvae to maximise the chance that some will encounter a carcass. In contrast, producing fewer, larger larvae is favourable at high population densities because larger beetles are better able to compete for carcasses (Otronen, 1988). Population density can therefore shift the optimum in the trade-off between offspring size and number, and the production of fewer eggs at high densities is likely to concomitantly reduce laying spread. This trade-off may also be shifted by age and carcass size: older females produce fewer but heavier larvae (*N. pustulatus*, Rauter *et al.*, 2010) and females lay fewer but larger eggs on smaller carcasses (Chapter 3, Botterill-James *et al*. 2017). If clutch size is reduced in these circumstances where larger offspring are favourable, it is likely that there will be a concurrent reduction in hatching spread (Chapter 3, Smiseth *et al*. 2008; Botterill-James *et al*., 2017) and hatching times may be less strongly skewed (Chapter 5, Smiseth *et al*., 2008).

High population density may cause intense competition for adult food (Steiger *et al*., 2007), resulting in poorer female nutritional condition, which constrains female egg production (Trumbo and Robinson, 2004; Steiger *et al*., 2007; Steiger, 2013). In addition to population density, female nutritional condition can be affected by carcass size. Females need to feed from the carcass to mature their ovaries (Wilson and Knollenberg, 1984), but the carcass will also provide the sole food resource for the larvae. Females may be in better nutritional condition on larger carcasses where they can eat more for themselves without depleting the carcass to the extent that there is insufficient food for the offspring once they hatch. In Chapter 3, I found that females breeding on smaller carcasses lay fewer eggs and because of this they produce clutches with smaller laying spreads. Nutritional condition directly influences hatching patterns as well as having an indirect effect through clutch size; females in better nutritional condition produce clutches with larger hatching spreads (Steiger, 2013).

The consequences of asynchronous hatching may feed back and affect hatching patterns in future generations. For example, I found that greater laying spread causes a reduction in the average mass of dispersing larvae (Chapter 2). This leads to a reduction in adult body size, which in turn affects laying patterns once these individuals themselves reproduce. Ultimately, hatching asynchrony is affected by multiple factors that interact with each other. Sexual conflict over parental care, the hurry-up hypothesis, female age and female inbreeding status affect each other or are affected by common influences such as carcass size and

population density. Some pathways have opposing influences on hatching asynchrony from the same initial potential cause, and the resulting degree of hatching asynchrony may therefore reflect the relative strengths of the relevant pathways.

### 6.12 *Concluding remarks*

My work highlights that there are many potential direct and indirect influences on hatching asynchrony that can interact in a complex manner to determine the ultimate hatching pattern. Hatching asynchrony can result from constraints on laying, but females also facultatively adjust laying patterns and can potentially use greater laying synchrony to compensate for other constraints on reproduction imposed by their state or breeding conditions. Asynchronous hatching has profound consequences for offspring growth and survival in *N. vespilloides* and is one of the most important maternal influences on offspring fitness.

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# Addendum to Chapter 2

This experiment was carried out by Oriane Carriot, Megan Golding and Matthieu Paquet with my assistance. I carried out these analyses.

## INTRODUCTION

In Chapter 2 I found that males remained longer with highly asynchronous broods while females deserted these broods earlier compared to synchronous broods. However, I found no evidence that females adjusted their laying patterns facultatively to manipulate males to provide more care by laying more asynchronously when the male was present. Placing the male on the carcass with the female and then removing him after 6 hours may not provide a sufficient cue to females that the male will not be present to assist with post-hatching care. I therefore carried out a similar experiment where females laid eggs either in the presence or absence of a male. In this experiment, pairs mated in a Petri dish for 24 hours prior to breeding and only females were placed on the carcass used to initiate breeding in the male absent treatment (Paquet and Smiseth, 2017). Thus, females in the male absent treatment were never accompanied by a male on the carcass. In Chapter 2, I also found that larval growth and survival would be increased if females lay more synchronously than they actually do on average. In light of this, I investigated whether there was a cost of laying synchronously which might explain why females produce laying patterns that do not appear optimal for the offspring.

## METHODS

### Study animals

The beetles used in this study were from a large outbred laboratory population maintained at the University of Edinburgh and were housed in individual transparent plastic boxes (124mm x 82mm x 22mm) from the day that they eclosed as adults. Beetles were kept at 20 ± 2°C and were fed small pieces of organic beef twice a week. All beetles were sexually mature, virgins and of prime reproductive age (12-17 days post-eclosion) at the start of the experiments.

### Experimental procedures

In all treatments, a virgin female and unrelated male were allowed to mate for 24 hours in a Petri dish (90mm x 12mm). I then weighed and transferred each female to a clear breeding box (170mm x 120mm x 60mm) containing 1cm of moist compost

and a previously frozen mouse carcass weighing 22.77g ± 1.62 (mean ± SD, supplied from Livefoods Direct Ltd, Sheffield, UK). Half of the females were accompanied by their male partners (*n* = 58) while the other half were transferred alone (*n* = 51). This allowed me to compare laying behaviour when the male is present or absent. I placed the breeding boxes on flat-bed scanners (Canon Canoscan 9000F Mark II, Canon Inc., Tokyo, Japan) and scanned the breeding boxes every hour throughout laying using Vuescan professional edition software (Hamrick Software, Sunny Isles Beach, FL, USA). After laying was complete but before larvae began to hatch, I weighed all of the females and returned them to their individual boxes. I fed them small pieces of organic beef twice a week and monitored them to estimate when they died. I then measured the pronotum width of each female. This allowed me to assess the cost of pre-hatching expenditure to the female without confounding effects of females providing post-hatching care. I analysed the scans in the manner described in Chapter 2 to calculate the total clutch size, delay until laying, laying spread, laying skew index and egg volume. Proportional mass change during laying was calculated for each female by subtracting the mass after laying from the initial mass and dividing this by the initial mass.

**Statistical analyses**

All analyses were carried out in R 3.3.1 (R Core Team, 2014). I tested for a difference in clutch size, egg volume and delay until laying between treatments (male presence and male absence) using Wilcoxon rank sum tests because these traits might influence laying patterns. I then carried out GLMs to test for an effect of male presence or absence on laying spread (Gaussian family, log link function) and laying skew (inverse Gaussian family, inverse link function). I selected model families and link functions based on graphical model validation, Fligner-Killeen tests for homogeneity of variance and AIC values. To facilitate analyses, I added 1 to each value of laying skew index to remove negative numbers. I first tested how laying spread or laying skew differed between treatments, and then investigated how this was affected by the delay until laying because this differed between treatments. To investigate the potential costs of producing certain laying patterns, I carried out a GLM to test how the proportional mass change of females during laying was affected by pronotum width, clutch size, laying spread, laying skew and the delay until laying (Gaussian family, identity link function). I then carried out a

GLM to test how lifespan was affected by laying spread, laying skew, the delay until laying and mass change (inverse Gaussian family, log link function).

**RESULTS**

*Do females change their laying patterns in the presence of a male?*

I found no difference in the number of eggs laid by females in the presence or absence of a male (W = 999, *P* = 0.560) or the volume of those eggs (W = 1473, *P* = 0.300). Females started to lay sooner when the male was present (W = 1726, *P* = 0.0004); on average, they took 16.11h to begin laying in the presence of a male and 27.48h when the male was absent.

Male presence had a significant effect on laying spread ($F_{1,91}$ = 5.22, *P* = 0.025); laying spread was greater when the male was present (mean 40.18h) than when the male was absent (mean 34.05h). This difference was no longer evident when controlling for the delay until laying ($F_{1,90}$ = 0.87, *P* = 0.353), which negatively affected laying spread ($F_{1,90}$ = 8.24, *P* = 0.005), suggesting that the greater laying spread of clutches produced in the presence of a male was due to laying commencing earlier in this treatment.

There was no significant difference in the laying skew index of clutches laid in the presence or absence of a male ($F_{1,91}$ = 1.71, *P* = 0.194). This was still the case when controlling for the delay until laying ($F_{1,90}$ = 0.03, *P* = 0.861). Females that commenced laying sooner after being placed with the carcass produced clutches where laying was less strongly skewed towards the beginning of the laying period ($F_{1,90}$ = 6.10, *P* = 0.015).

*Does the cost of laying for females differ depending on their laying pattern?*

Females gained proportionally more mass when they started laying later ($F_{1,36}$ = 7.63, *P* = 0.009) and larger females gained more mass ($F_{1,36}$ = 4.15, *P* = 0.049). Female mass change was not significantly affected by laying spread ($F_{1,36}$ = 0.05, *P* = 0.818), laying skew ($F_{1,36}$ = 0.005, *P* = 0.943) or clutch size ($F_{1,36}$ = 1.21, *P* = 0.279).

Female lifespan was affected by laying spread ($F_{1,35}$ = 5.93, *P* = 0.020) and the delay until laying ($F_{1,35}$ = 5.28, *P* = 0.028); females that started to lay sooner and produced clutches with a smaller laying spread lived longer. Lifespan was not significantly affected by female mass change ($F_{1,35}$ = 0.29, *P* = 0.595) or laying skew ($F_{1,35}$ = 3.10, *P* = 0.087).

**DISCUSSION**

Females produced clutches with a greater laying spread when they were accompanied by the male on the carcass, but this was due to laying starting earlier when the male was present. One potential explanation for this is that males assist with carcass preparation (Bartlett, 1988), which may allow females to focus on feeding from the carcass to mature her oocytes and begin to lay sooner after encountering the carcass. Females lived longer if they had a shorter delay until laying and produced clutches with a smaller laying spread. This could reflect that there is a cost of producing clutches with a greater laying spread, in which case delaying the start of laying should be associated with increased in lifespan. Alternatively, it might reflect that females of higher quality are able to lay more synchronously and start laying sooner, and that these higher-quality females also live longer.

# Addendum to Chapter 5

## INTRODUCTION

In Chapter 5 I investigate the effect of female age at breeding on the distribution of laying times within the clutch. I allowed females aged 11 to 79 days post-eclosion to breed in clear boxes containing 1cm of compost and scanned the boxes every hour during laying. I counted the number of eggs on each scan and subtracted the number on the previous scan to calculate the number of eggs that were laid each hour. I used this information to determine the number of hours since the female was placed in the box with the carcass until each egg was laid. I assigned a value $t_{ij}$ to each egg *ij* in clutch *i* representing the number of hours after the female encountered the carcass until that egg was laid, referred to as $d_1$. I subtracted the mean time of laying for each clutch, $t_i$. from the value for each egg, and calculated the second-and third-order deviations from the clutch-mean, $(t_{ij}-t_{i.})^2$, referred to as $d_2$, and $(t_{ij}-t_{i.})^3$, referred to as $d_3$. In Chapter 5, I carried out a multivariate analysis to explain how changes in female age, clutch size and carcass size affect $d_1$, $d_2$ and $d_3$. I found that all laying metrics decreased with female age: older females laid eggs sooner after encountering the carcass and produced clutches with less variation in the timing of laying and less positive laying skew than young females. Because $d_2$ and $d_3$ are related, I carried out similar analyses at the level of the clutch rather than individual eggs to determine whether the effects of female age follow the same pattern. The metrics I used were the mean time of laying for the clutch, laying spread and laying skew index (the same metrics used throughout this thesis).

## METHODS

### Experimental procedures

Data were collected as described in Chapter 5. I calculated mean laying time by taking the mean of $d_1$ for each clutch. I used the following formula to calculate a laying skew index (as described in Section 1.8 of the General Introduction) for each clutch: $\Sigma((t_i - t_m)/t_m)p_i$, where $p_i$ is the proportion of the total clutch laid each hour, $t_i$ is the time interval starting from the initiation of oviposition and $t_m$ is the middle of the laying period. I calculated laying spread as the time between the laying of the first and last eggs.

**Statistical analyses**

Analyses were carried out in ASReml version 4.1 (Gilmour *et al*., 2015). I fit a multivariate model including female age, clutch size and carcass size as fixed effects. I included linear and quadratic effects of carcass size, using the *pol* function. The model differed from that in Chapter 5 in that the response variables were mean laying time, laying spread and laying skew index rather than $d_1$, $d_2$ and $d_3$. I calculated *P*-values from *z*-scores obtained by dividing the estimated effect sizes for each laying metric by their estimated standard errors.

**RESULTS**

I found that female age at breeding had a significant effect on the clutch-specific metrics of laying times (Table 1). The mean timing of laying of a clutch decreased by 1.17h for each additional day of female age; older females laid significantly earlier ($z$ = -23.65, $P$ <0.001). Laying spread also decreased with female age ($z$ = -2.20, $P$ = 0.028). The time to between the start and end of laying was reduced by 0.10h for each additional day of female age. Female age at breeding did not significantly affect laying skew index ($z$ = -1.10, $P$ = 0.309).

Table 1: Model output from a multivariate analysis investing how changes in female age, clutch size and carcass size affect mean laying time, laying spread and laying skew index.

| | | Mean laying time | | | Laying spread | | | Laying skew index | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Variables | | Effect size (SE) | *z* | *P* | Effect size (SE) | *z* | *P* | Effect size (SE) | *z* | *P* | *F* test | *P* |
| Female age | | -1.168 (0.049) | -23.65 | <0.001 | -0.100 (0.046) | -2.20 | 0.028 | -0.001 (0.001) | -1.10 | 0.309 | $F_{3,128.0} = 4.75$ | 0.004 |
| Clutch size | | 0.333 (0.096) | 3.48 | <0.001 | -0.327 (0.088) | -3.71 | <0.001 | -0.004 (0.002) | -2.64 | 0.008 | $F_{3,128.0} = 11.18$ | <0.001 |
| Carcass size | Linear | 3.068 (7.970) | 0.38 | 0.700 | 5.690 (7.356) | 0.77 | 0.439 | 0.138 (0.137) | 1.01 | 0.313 | $F_{6,169.2} = 2.29$ | 0.038 |
| | Quadratic | -9.667 (5.125) | -1.89 | 0.059 | 8.171 (4.731) | 1.73 | 0.084 | 0.223 (0.088) | 2.53 | 0.011 | | |

**DISCUSSION**

In accordance with the egg-level analyses, I found that older females laid eggs sooner after encountering the carcass and produced clutches with less variation in the timing of laying: mean laying time and laying spread were reduced in clutches produced by older females. However, while $d_3$ decreased with age, there was no significant effect of female age at breeding on laying skew index. This apparent discrepancy between egg-level and clutch-level analyses is likely explained by the way the metrics are calculated. Laying skew index is the sum of the proportion of the clutch laid in each time interval (i.e., each hour) relative to middle of the laying period, while $d_3$ is the cubed distance from the mean timing of laying for the clutch. For most clutches, the mean laying time is earlier than the middle of the laying period because there is usually a positively skewed distribution of laying times within the clutch. The last-laid eggs have very large $d_3$ values because they are the furthest from the mean. In clutches produced by older females, the distribution of laying times is less strongly asymmetrical and this change is mainly driven by a reduction in the tail of the distribution. Because the distribution of laying times is less positively skewed, the last-laid eggs are not so far from the mean time of laying of the clutch. This difference is amplified by the values being cubed and therefore there is a significant decrease in $d_3$. In contrast, the reduced tail to the distributions of laying times in clutches produced by older females compared to younger females is insufficient to result in a significant decrease in laying skew index. The last time intervals of the laying period represent a very small proportion of the total clutch. Despite the time intervals further from the middle of the laying period being more heavily weighted in the skew index calculation, the contribution of the eggs laid during the last few hours of the laying period is relatively small. Additionally, the models for clutch-specific metrics also have less power than egg-level models due to smaller sample sizes.

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# Asynchronous hatching provides females with a means for increasing male care but incurs a cost by reducing offspring fitness

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## Abstract

In species with biparental care, sexual conflict occurs because the benefit of care depends on the total amount of care provided by the two parents while the cost of care depends on each parent's own contribution. Asynchronous hatching may play a role in mediating the resolution of this conflict over parental care. The sexual conflict hypothesis for the evolution of asynchronous hatching suggests that females adjust hatching patterns in order to increase male parental effort relative to female effort. We tested this hypothesis in the burying beetle *Nicrophorus vespilloides* by setting up experimental broods with three different hatching patterns: synchronous, asynchronous and highly asynchronous broods. As predicted, we found that males provided care for longer in asynchronous broods whereas the opposite was true of females. However, we did not find any benefit to females of reducing their duration of care in terms of increased lifespan or reduced mass loss during breeding. We found substantial negative effects of hatching asynchrony on offspring fitness as larval mass was lower and fewer larvae survived to dispersal in highly asynchronous broods compared to synchronous or asynchronous broods. Our results suggest that, even though females can increase male parental effort by hatching their broods more asynchronously, females pay a substantial cost from doing so in terms of reducing offspring growth and survival. Thus, females should be under selection to produce a hatching pattern that provides the best possible trade-off between the benefits of increased male parental effort and the costs due to reduced offspring fitness.

## Introduction

Sexual conflict, defined as a divergence in the evolutionary interests of individuals of the two sexes (Parker, 2006), is now recognized as ubiquitous in a variety of contexts, including mating and parental care (Arnqvist & Rowe, 2005). In species with biparental care, there is conflict over how much care each parent should provide because the benefit of care depends on the total amount of care provided by the two parents while the cost of care depends on each parent's own contribution (Lessells, 2012). As a consequence of this conflict, each parent is expected to minimize its costs of care by shifting as much of the workload as possible over to its partner (Trivers, 1972). One mechanism that might play a role in mediating the resolution of sexual conflict over parental care is asynchronous hatching, which occurs when the offspring from a single reproductive event hatch over an extended period of time (Clark & Wilson, 1981). The sexual conflict hypothesis for the evolution of asynchronous hatching suggests that females adjust hatching patterns in order to increase male parental effort relative to female effort (Slagsvold & Lifjeld, 1989). Female birds can control hatching patterns by altering the timing of the onset of incubation: the brood hatches synchronously if the onset of incubation occurs after the clutch has been completed, while it

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hatches asynchronously if incubation begins before the last egg has been laid (Clark & Wilson, 1981). The initial version of this hypothesis proposed that the female benefits from hatching the young more asynchronously by making the male start feeding the young as soon as the earliest offspring hatch such that he continues feeding for longer than with a synchronous brood (Slagsvold & Lifjeld, 1989). However, a later version (sometimes termed the 'exploitation of mate hypothesis'; Slagsvold *et al.*, 1995), suggested that the female benefits from hatching the young more synchronously because the male has to contribute more effort to prevent the brood from starving due to the peak in demand of each offspring occurring simultaneously.

The hypothesis that asynchronous hatching plays a role in mediating the resolution of sexual conflict over parental care predicts that the female should gain a fitness benefit from adjusting hatching patterns by increasing her partner's contribution towards parental care, thereby allowing her to reduce her own contribution. Previous studies on birds, which have tested this hypothesis by manipulating the degree of hatching asynchrony, have found mixed evidence. In support of the exploitation of mate hypothesis, Slagsvold (1997) found that males contributed more care towards synchronous broods whereas females reduced their contribution relative to asynchronous broods. Other studies have been unable to detect a consistent difference in male feeding rate between synchronous and asynchronous broods (Hillström, 1992; Amundsen, 1993; Hébert & Sealy, 1993; Stoleson & Beissinger, 1997). Furthermore, Slagsvold *et al.* (1994) found that synchronous hatching increased female survival in the subsequent year whereas asynchronous hatching increased male survival. In contrast, Stoleson & Beissinger (1997) found no difference in survival of male and female parents raising synchronous or asynchronous broods. Asynchronous hatching and biparental care are not unique to birds, but also occur in some insects (Nalepa, 1988; Müller & Eggert, 1990) and reptiles (While, 2007). Thus, to improve our understanding of how asynchronous hatching contributes towards the resolution of sexual conflict over parental care, there is now a need to extend this work to nonavian systems.

Burying beetles of the genus *Nicrophorus* exhibit hatching asynchrony similar to that of many altricial birds (Müller & Eggert, 1990; Smiseth *et al.*, 2006; Takata *et al.*, 2015). These beetles breed on small vertebrate carcasses, which are buried underground (Scott, 1998). Females lay eggs in the surrounding soil (Pukowski, 1933). In contrast to birds, burying beetles do not incubate the eggs. Instead, the asynchronous hatching pattern is determined by the period of time over which the eggs are laid, which is termed 'laying spread' (Smiseth *et al.*, 2006; Takata *et al.*, 2015) and the extent to which laying is skewed towards the earlier part of the laying period, which is termed 'laying skew' (Smiseth *et al.*, 2008) Thus, females can control the hatching pattern simply by adjusting laying spread and laying skew. In *Nicrophorus vespilloides* (Herbst), the mean interval between the hatching of the first and last larvae of a brood (i.e. hatching spread) is 30 h. Given that the larvae disperse into the soil around 6 days after hatching, the hatching spread is considerable relative to the amount of time the larvae spend on the carcass (Smiseth *et al.*, 2006). *Nicrophorus vespilloides* exhibits facultative biparental care (Bartlett, 1988). Either parent is capable of raising the brood alone, providing the opportunity for one parent to desert the brood and leave the other to care for the offspring (Bartlett, 1988). Parents provide care by preparing the carcass, defending it and the brood from predators and conspecifics, applying antimicrobials to the carcass, and provisioning the larvae with predigested carrion (Eggert *et al.*, 1998; Rozen *et al.*, 2008; Walling *et al.*, 2008; Arce *et al.*, 2012). Sexual conflict over parental care occurs if parents benefit from reducing their investment in the current brood by increasing their survival and future reproductive success or by increasing the chances of finding another mate during the breeding season (Maynard Smith, 1977). *Nicrophorus vespilloides* appears to fulfil these criteria because there is a cost associated with providing care (Ward *et al.*, 2009) and both sexes can breed more than once in a season (Bartlett & Ashworth, 1988) without any delay after rearing a brood (Scott & Traniello, 1990).

We conducted two experiments to test the sexual conflict hypothesis in *N. vespilloides*. Previous work on the resolution of sexual conflict over parental care highlights the distinction between evolutionary and facultative responses when studying how a focal parent adjusts its care to a change in the partner's workload, termed 'sealed-bids' and 'negotiation', respectively (Lessells, 2012). Thus, in Experiment 1, we tested whether females facultatively adjust hatching patterns in order to manipulate males to increase their contribution to parental care. Given that biparental care in *N. vespilloides* is facultative, females may adjust hatching patterns depending on whether the male partner is present or absent at the start of breeding. The presence or absence of the male might provide females with a reliable cue as to whether a male is likely to assist in providing care for the larvae once the eggs have hatched. We recorded the timing of oviposition of females laying in the presence or absence of a male using scanners to minimize interference while females lay eggs. If females facultatively adjusted hatching patterns, we predicted that laying spread would differ when the male was present compared to when he was removed. In Experiment 2, we tested whether variation in the hatching pattern influences the male's contribution towards parental care and whether there is a benefit to females should the male make a greater contribution to parental care.

Burying beetles do not differentiate between their larvae and larvae produced by other females as long as the larvae are introduced after their own eggs have hatched (Müller & Eggert, 1990). This allows us to use a cross-fostering design where we provided females with foster broods of a standardized brood size and a particular degree of asynchrony. We set up broods with three different hatching patterns (synchronous, asynchronous and highly asynchronous broods) and recorded how long each parent remained with the brood as a proxy for the amount of parental care. We assessed the fitness consequences for the parents by measuring effects on the survival and growth of the larvae and on the longevity and mass change of the parents. If asynchronous hatching plays a role in mediating the resolution of sexual conflict over parental care, we predicted that females would reduce their duration of care in broods with a greater hatching spread, with a corresponding increase in male care. We expected that reducing the amount of effort they invest in parental care would lead to a fitness benefit for females, such as an increase in the female's lifespan or a reduction in her loss of body mass during breeding.

## Materials and methods

### Study animals

The beetles used in this study were from an outbred laboratory population maintained at the University of Edinburgh. Beetles were housed individually in clear plastic boxes (124 × 82 × 22 mm or 110 × 110 × 33 mm). They were kept at 20 ± 2 °C (mean ± range) under constant lighting and were fed small pieces of organic beef twice a week. The beetles were aged 18–27 days post-eclosion at the start of the experiments.

### Experimental procedures

*Experiment 1*

To determine whether females facultatively adjust laying patterns to increase the male's contributions towards care, we allowed females to lay eggs either in the presence or the absence of a male. We paired unrelated virgin males and females and placed them in a clear breeding box (17 × 12 × 6 cm) containing 1 cm of compost. We supplied each pair with a mouse carcass weighing 19.56–22.27 g (previously frozen, supplied from Livefoods Direct Ltd, Sheffield, UK), which is within the range of vertebrate carcasses utilized by beetles in the wild (range: 1–37 g; Müller *et al.*, 1990; Smiseth & Moore, 2002). We removed the male from half of the boxes after 6 h, while leaving the male with the female in the remaining boxes (male present $n = 26$, male absent $n = 24$). Previous work suggests that parents respond to the absence of their partner within 45 min of removal (Steiger & Müller, 2010). Thus, given that the first eggs were laid after an average of 24 h after pairing, females had ample time (on average 18 h) to notice the male's absence before they began oviposition. Eggs are visible at the bottom of the breeding box and can be seen on images obtained by placing the boxes on flat-bed scanners (Canon Canoscan 9000F Mark II, Canon Inc., Tokyo, Japan). In the small amount of soil used, the visible number of eggs is very similar to the actual clutch size (Monteith *et al.*, 2012). We scanned the breeding boxes every hour using Vuescan professional edition software (Hamrick Software, Sunny Isles Beach, FL, USA) until after the completion of oviposition. From the scanned images, we counted the number of new eggs laid each hour to determine the laying spread (the time between the first and last egg being laid) and the clutch size. We calculated a laying skew index (based on the hatching skew index of Smiseth *et al.*, 2008) for each brood using the following formula: $\Sigma((t_i - t_m)/t_m)p_i$, where $p_i$ is the proportion of the total clutch laid each hour, $t_i$ is the time interval starting from the initiation of oviposition and $t_m$ is the middle of the laying period. To account for possible effects due to female and male body size, we also measured the pronotum widths of the parents using a Mitutoyo Absolute Digimatic calliper with a precision of 0.01 mm. We set up 86 pairs initially, but in analyses we excluded all pairs where either the eggs did not hatch ($n = 26$) or there were technical problems with the scanner ($n = 10$).

*Experiment 2*

In order to investigate the influence of hatching patterns on the duration of care provided by males and females, we used a 2 × 3 fully factorial design with male presence vs. absence and hatching spread (synchronous, asynchronous or highly asynchronous hatching) as the main factors. To set up the broods, we weighed virgin beetles, paired females with unrelated males, and placed each pair in a clear breeding box (17 × 12 × 6 cm) containing 1–2 cm of compost. We provided each pair with a mouse carcass weighing 19.37–22.22 g (previously frozen, supplied from Livefoods Direct Ltd). In half of the trials, we removed the male 6 h after we provided the pair with a carcass, which is before the female had initiated egg laying. In the remaining trials, the male was left with the female during egg laying. In the interval between the end of egg laying and the start of hatching (i.e. 54–66 h after pairing), we moved the remaining parents and the prepared carcass to a new box with fresh soil, while the eggs were left to develop in the original box. The larvae hatching from these eggs were then used to generate experimental foster broods. To ensure that we had an ample supply of foster larvae to generate the experimental broods, we set up additional donor pairs for breeding on the same day as the experimental pairs. We also set up some additional donor pairs over the

consecutive 2 days. As soon as possible after their own larvae began to hatch, we provided breeding beetles with experimental foster broods that differed with respect to hatching spread (Smiseth & Morgan, 2009). The experimental broods were comprised of larvae that were unrelated to the foster parents and that were derived from up to four different donor females. The larvae were newly hatched and had not previously received any parental care from other individuals. Caring parents always received a total of 20 larvae, which is similar to the mean brood size of 21 larvae in this species (Smiseth & Moore, 2002). We weighed the larvae before placing them on the carcass as a measure of prenatal maternal investment. We generated synchronous broods by providing parents with 20 larvae at the same time. We generated asynchronous broods by providing parents with 10 larvae initially and then an additional 10 larvae 24 h later. Finally, we generated highly asynchronous broods by providing parents with 10 initial larvae followed by 10 additional larvae 48 h later. Thus, synchronous broods had a hatching spread of 0 h, while asynchronous broods had a hatching spread of 24 h and highly asynchronous broods had a hatching spread of 48 h. This is within the natural variation of hatching spread, which can extend up to 56 h in this species with a mean of around 30 h (Smiseth *et al.*, 2006). The total sample size in the experiment was $n = 126$. The sample sizes for each treatment were as follows: $n = 20$ for synchronous brood with male present, $n = 22$ for synchronous brood with male absent, $n = 20$ for asynchronous brood with male present, $n = 22$ for asynchronous brood with male absent, $n = 22$ for highly asynchronous brood with male present and $n = 20$ for highly asynchronous brood with male absent.

We used the amount of time that each parent spent with the brood from the arrival of the first larvae as a proxy for the amount of care they provided (Boncoraglio & Kilner, 2012; Smith *et al.*, 2014). We checked each box twice a day (at 09:00 and 17:15 h) to determine whether the parent was present or absent from the brood chamber. If a parent was absent in two consecutive observations, we regarded it as having deserted the brood (Smith *et al.*, 2014). Once the deserting parent had been removed from the box, we weighed it to record its post-breeding body mass. If parents did not desert the brood before larval dispersal (defined as when the majority of larvae left the carcass), we weighed and removed them at the time when the larvae dispersed from the carcass. We placed all parents in individual boxes upon removal from the breeding box, and fed them small pieces of organic beef twice a week. We recorded the number of larvae dispersing from each brood and weighed the entire brood to obtain the total dispersing brood mass, from which we calculated the average larval mass. We then placed the larvae in a box (17 × 12 × 6 cm) filled with soil and allowed them to eclose. As keeping all offspring would amount to an excessive workload, we randomly selected one male and one female offspring from each brood upon eclosion and retained them to record potential effects on lifespan. We recorded the sex and pronotum width of the other offspring. We checked parents and retained offspring at least three times a week to obtain the approximate age of death, and measured their pronotum widths using a Mitutoyo Absolute Digimatic calliper.

### Statistical analyses

We carried out the statistical analyses in R (R Core Team, 2014). We selected model families and link functions based on graphical model validation and AIC values where appropriate. We carried out model refinement through backwards stepwise deletion using the drop1 function ($P$-values based on $F$ or Chi statistics). To analyse the results of Experiment 1, we constructed generalized linear models to investigate the effect of male removal on laying spread (Gamma family, inverse link function) and laying skew (Gaussian family, identity link function). We used Kendall's Tau correlation to test for a correlation between laying spread and laying skew. We constructed generalized linear models to investigate the influence of hatching pattern on parental care and aspects of parent and offspring fitness studied in Experiment 2. Table 1 shows the full models and the model family and link function used in each model. We used Kendall's Tau correlation to test for a correlation between male presence and female presence. We used Wilcoxon signed ranks test to determine whether female presence was affected by removal of the male. We also used Wilcoxon signed ranks tests to compare female age at death and male age at death between treatments where the male was removed or was allowed to remain with the brood. Finally, we compared total parental presence between hatching patterns using a Kruskal–Wallis test.

## Results

### Experiment 1

In contrast to what we predicted if females facultatively adjusted their egg laying to the presence or absence of the male, the removal of the male before oviposition did not affect average laying spread ($F_{1,48} = 0.09$, $P = 0.768$). Laying skew was also not affected by male removal ($F_{1,48} = 2.60$, $P = 0.114$), and there was no correlation between laying spread and laying skew ($z = 0.30$, $P = 0.763$). Laying spread was greater for larger clutches ($F_{1,48} = 21.57$, $P < 0.0001$), and there was a nonsignificant trend towards a greater laying spread when oviposition commenced earlier ($F_{1,48} = 4.00$, $P = 0.0514$).

**Table 1** Summary of generalized linear models showing all terms included in the full model, and model families and link functions.

| Response variable | Full model | Family | Link function |
|---|---|---|---|
| Female presence | Hatching pattern + Carcass mass + Female body size + Female age | Quasi | $1/\mu^2$ |
| Male presence | Hatching pattern + Carcass mass + Male body size + Male age | Quasi | Identity |
| Parent proportional mass change | Hatching pattern + Sex + Sex : Hatching pattern + Time present + Carcass mass + Initial mass | Gaussian | Identity |
| Female lifespan | Hatching pattern + Time present + Body size + Age | Gamma | Identity |
| Male lifespan | Hatching pattern + Time present + Body size + Age | Gamma | Identity |
| Number of larvae dispersing | Hatching pattern + Total parental presence + Male presence + Larval development time + Carcass mass + Initial mass | Poisson | Identity |
| Mean larval mass at dispersal | Hatching pattern + Male presence + Hatching pattern : Male presence + Total parental presence + Larval development time + Carcass mass | Inverse Gaussian | Identity |
| Mean offspring pronotum width | Hatching pattern + Male presence + Hatching pattern : Male presence + Carcass mass | Gaussian | Inverse |
| Standard deviation in offspring pronotum width | Hatching pattern + Male presence + Hatching pattern : Male presence + Carcass mass | Inverse Gaussian | Log |
| Duration of larval development | Hatching pattern + Male presence + Carcass mass | Gaussian | Identity |
| Offspring age at death | Hatching pattern + Male presence + Body size + Sex + Total parental presence | Gamma | Inverse |

## Experiment 2

Consistent with what we predicted, the hatching pattern had a significant effect on the amount of time that the male was caring for the brood (the number of observations the parent was present out of total number of observations when there were larvae on the carcass) ($t_{58} = 3.18$, $P = 0.0024$). The male remained for longest when caring for highly asynchronous broods (Fig. 1a). In contrast, the female deserted highly asynchronous broods sooner ($F_{1,60} = 5.41$, $P = 0.0234$, Fig. 1b). Although the amount of time the male was present was highly negatively correlated with the amount of time the female was present ($z = -3.27$, $P = 0.0011$), the amount of time the female was present was not affected by the removal of the male before oviposition ($W = 2246$, $P = 0.171$). This suggests that the male responds by delaying his desertion of the brood when the female deserts first, while the female's decision was not affected by male desertion. Furthermore, the male responded differently to female desertion depending on the hatching pattern (interaction between hatching pattern and amount of time females provided care: $F_{1,58} = 9.68$, $P = 0.0029$); the male responded more strongly to female desertion when he was caring for highly asynchronous broods. Despite this, the total amount of presence by male and female parents was similar across all three hatching patterns ($\chi^2 = 4.74$, $P = 0.0934$). There was no effect of female pronotum width ($F_{1,60} = 3.01$, $P = 0.0938$) or male pronotum width ($F_{1,58} = 0.00$, $P = 0.997$) on the duration of care provided.

In contrast to what we predicted, we found no evidence that a reduction in the duration of female care led to an increase in the female's own lifespan or a reduction in her loss in body mass during breeding. The female's adult lifespan (number of days from eclosion to death) was not affected by the hatching pattern ($F_{1,123} = 1.16$, $P = 0.283$). Furthermore, female lifespan was not affected by the absolute duration of time the female spent with the brood ($F_{1,123} = 0.16$, $P = 0.695$). Similarly, male lifespan was not affected by the hatching pattern ($F_{1,60} = 0.38$, $P = 0.541$) or the amount of time spent with the brood ($F_{1,60} = 0.06$, $P = 0.814$). Females had a significantly longer lifespan than the males ($W = 4492$, $P < 0.0001$). Female lifespan was not affected by whether the male was present or absent ($W = 1934$, $P = 0.931$), whereas males survived slightly longer when allowed to remain with the brood ($W = 1519$, $P = 0.0322$). We found that most parents (95% of all parents) gained mass during the breeding attempt. Mass change was not affected by hatching pattern ($F_{1,185} = 0.41$, $P = 0.521$), sex ($F_{1,185} = 0.18$, $P = 0.670$), or the interaction between sex and hatching pattern ($F_{1,185} = 0.01$, $P = 0.907$). However, mass change was greater when parents remained with the brood for longer ($F_{1,185} = 6.44$, $P = 0.0120$) and was also greater for parents that initially had a lower body mass ($F_{1,185} = 11.15$, $P = 0.0010$).

Hatching patterns influenced larval survival as a greater number of larvae survived to dispersal in synchronous or asynchronous broods than in highly asynchronous broods ($\chi_{1,123} = 12.76$, $P = 0.0004$, Fig. 2). Larval survival was not affected by male removal ($\chi_{1,123} = 0.66$, $P = 0.418$). However, the number of surviving larvae was greater when the larvae had a greater initial mass at the time when they were introduced to the carcass ($\chi_{1,123} = 26.40$, $P < 0.0001$). The duration of larval development was also affected by hatching pattern ($F_{1,124} = 22.12$, $P < 0.0001$) as highly

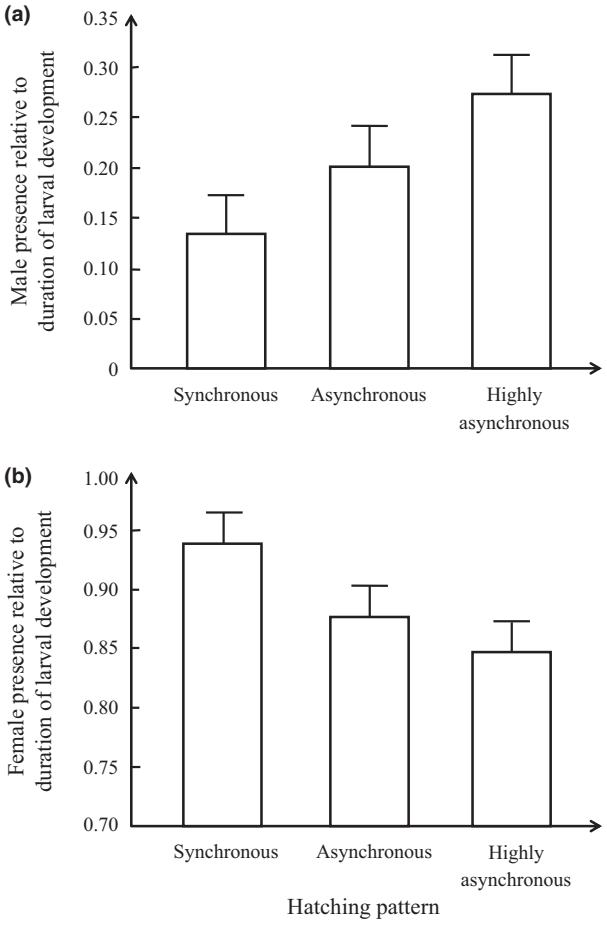


**Fig. 1** Duration of time spent with the brood as a proportion of the total time larvae were on the carcass for synchronous, asynchronous and highly asynchronous broods. (a) Mean duration of male presence with the brood. (b) Mean duration female presence with the brood. Error bars indicate ± 1 SE.

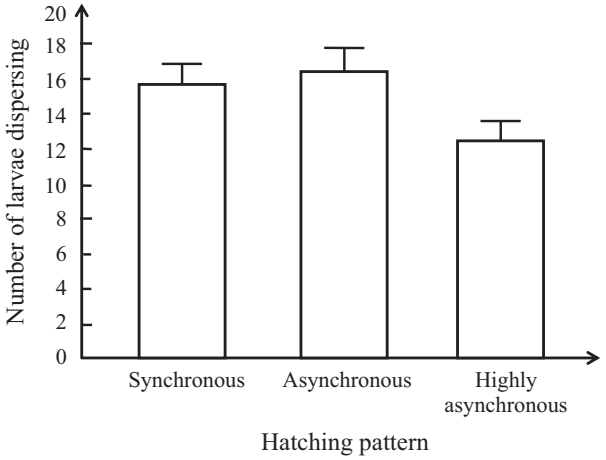


**Fig. 2** Mean number of larvae dispersing from the carcass for synchronous, asynchronous and highly asynchronous broods. Error bars indicate ± 1 SE.

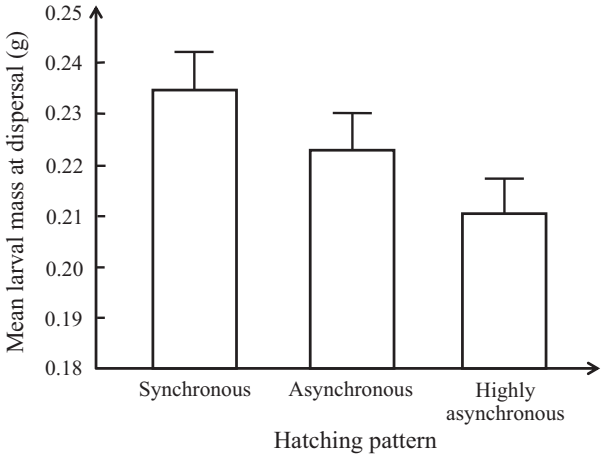


**Fig. 3** Mean mass of a larva at dispersal (total brood mass divided by number of larvae) for synchronous, asynchronous and highly asynchronous broods. Error bars indicate ± 1 SE.

asynchronous broods took on average 0.63 days longer to reach dispersal than synchronous broods. Offspring lifespan was not affected by hatching pattern ($F_{1,248} = 0.80$, $P = 0.371$), male removal ($F_{1,248} = 0.021$, $P = 0.886$), the total duration of parental care ($F_{1,248} = 0.29$, $P = 0.588$), the offspring's sex ($F_{1,248} = 3.28$, $P = 0.071$), or offspring pronotum width ($F_{1,246} = 0.33$, $P = 0.565$). The mean mass of a larva at dispersal decreased with increasing hatching spread ($F_{1,123} = 36.05$, $P < 0.0001$, Fig. 3) and increased with increasing total duration of parental presence ($F_{1,123} = 14.63$, $P = 0.0002$). However, mean larval mass was not affected by male removal ($F_{1,123} = 0.16$, $P = 0.694$). Similarly, the mean pronotum width of the offspring from each brood also decreased with increasing hatching spread ($F_{1,124} = 22.50$, $P < 0.0001$) and was not affected by male removal, although there was a nonsignificant trend towards greater offspring pronotum widths when the male was allowed to remain with the brood ($F_{1,124} = 3.91$, $P = 0.0502$). The standard deviation in offspring pronotum width increased with increasing hatching spread ($F_{1,124} = 79.10$, $P < 0.0001$).

## Discussion

In Experiment 1, we found that laying spread and laying skew were similar regardless of whether the male was experimentally removed or allowed to remain with the female during oviposition. This finding suggests that females do not adjust hatching patterns facultatively in response to male removal in *N. vespilloides*, and thus that the degree of asynchronous hatching represents an

evolutionary response to sexual conflict over parental care. We are unaware of any evidence from the literature showing that females adjust hatching patterns facultatively in response to the absence or removal of the male. The absence of evidence for facultative responses in *N. vespilloides* and birds might reflect that there has not been strong selection on females to adjust hatching patterns depending on whether the male is present or absent, possibly reflecting that females normally are assisted by a male partner (Scott, 1998; Cockburn, 2006). If the hatching pattern is an evolutionary response to sexual conflict over parental care, we might expect the optimal hatching pattern for the female to depend on male parental effort and the optimal parental effort for the male to depend on the hatching pattern. The outcome of this co-evolutionary process might be for females to evolve a hatching pattern that is associated with an evolutionary increase in male parental effort to relative to female parental effort.

In Experiment 2, we found that males remained for longer when caring for highly asynchronous broods than when caring for asynchronous and synchronous broods, while females in contrast deserted earlier when caring for highly asynchronous broods. This difference between males and females is consistent with the prediction of the sexual conflict hypothesis (Slagsvold & Lifjeld, 1989), and suggests that females could increase male contributions to parental care by laying the eggs more asynchronously. We suggest two possible mechanisms for the observed effect of hatching pattern on the duration of paternal care. Firstly, males may prolong their involvement in care when caring for highly asynchronous broods in response to the female deserting earlier. In support of this suggestion, we found that males remained with the brood for longer when their partner deserted earlier, and similar results showing that males adjust their contribution to the absence of the female have been found in previous studies on the amount of care (Fetherston *et al.*, 1994; Smiseth & Moore, 2004; Rauter & Moore, 2004; Smiseth *et al.*, 2005; Suzuki & Nagano, 2009; Smith *et al.*, 2014). We found that males responded more strongly to female desertion in highly asynchronous broods, but that the total duration of parental care was similar across all hatching patterns. Males may respond by staying for longer should the female desert early on to prevent conspecifics from usurping the carcass and killing the brood (Trumbo, 1990). Secondly, our results may reflect that males reduce their effort later if they contributed more towards care early on (Amundsen, 1999). Smiseth & Morgan (2009) found that the peak in brood demand is significantly higher in synchronous broods than in highly asynchronous broods. Thus, males may initially contribute more towards parental care when caring for synchronous broods while there is a high demand, and may desert the brood earlier as a consequence of their higher initial contribution. If so, males might potentially be contributing a similar amount of care towards synchronous and asynchronous broods by remaining for longer but providing lower levels of care in the latter broods. We have no information on the amount of care given that we used residency time as a proxy for parental effort instead of behavioural observations. Thus, further work is now needed to examine the mechanisms whereby hatching patterns influence the duration of paternal care.

We found that the duration of female care was not influenced by the removal of the male before larvae were present on the carcass, suggesting that females do not adjust their timing of desertion in response to male behaviour. Similar results showing that females do not respond to male removal have been found in previous studies on *N. vespilloides* (Smiseth *et al.*, 2005) and the closely related *Nicrophorus orbicollis* (Rauter & Moore, 2004). We also found that females deserted highly asynchronous broods earlier than asynchronous or synchronous broods. This finding might reflect that females value highly asynchronous broods less highly given that we also found that these broods produce fewer and smaller surviving larvae. Previous work shows that females adjust their provisioning behaviour in response to changes in the demand of the older larvae in the brood rather than the entire brood (Smiseth & Morgan, 2009). Thus, females may be more sensitive to the requirements of older larvae, in which case they might desert the brood depending on the age of the older larvae in the brood rather the average age of the brood. The greater duration of female care seen in synchronous broods with 20 older larvae than in asynchronous and highly asynchronous broods with 10 older larvae is consistent with this suggestion. It is not known whether males are more responsive to the needs of older larvae than younger larvae, but given that they provide less care overall than females, they may not be under strong selection to discriminate between different-aged larvae. Further work is needed to examine whether there is a difference in how males and females respond to the demand of the older larvae relative to the entire brood.

Our study shows that asynchronous hatching had detrimental effects on the offspring's fitness. Firstly, we found that larval survival was lowest in highly asynchronous broods, as previously reported by Smiseth & Morgan (2009). This finding shows that high levels of hatching asynchrony increase the offspring's mortality. Secondly, we found that mean larval mass at dispersal and offspring pronotum width at eclosion were lower in highly asynchronous broods, which contrasts with previous studies reporting no effect of hatching patterns on larval mass at dispersal (Smiseth *et al.*, 2008; Smiseth & Morgan, 2009). Such reductions in offspring size should have detrimental fitness consequences given that smaller offspring develop into smaller adults (Lock *et al.*, 2004), and that smaller adults are less likely to be

successful in competition for breeding resources (Otronen, 1988; Scott & Traniello, 1990; Trumbo, 1991; Robertson, 1993). Thirdly, we found that the duration of larval development was longer for highly asynchronous broods, potentially increasing the vulnerability of the brood to predation or infanticidal intruders. The detrimental effects of asynchronous hatching on offspring survival and growth are likely to be the outcome of asymmetric sibling competition caused by asynchronous hatching. Smiseth *et al.* (2007) found that older larvae in asynchronous broods grow better than younger larvae as long as the parents provide care, suggesting that parental care somehow exacerbates asymmetric sibling competition. Previous work on burying beetles suggests that older larvae consistently have higher survival and greater body mass than younger larvae regardless of hatching spread or skew (Takata *et al.*, 2014), reflecting that they receive more parental care (Smiseth *et al.*, 2007; Smiseth & Moore 2008; Takata *et al.*, 2013; Andrews & Smiseth, 2013). Similar detrimental effects of asymmetric sibling competition on offspring survival and growth have also been reported in birds (Clark & Wilson, 1981). These detrimental fitness consequences of asynchronous hatching have important consequences for the sexual conflict hypothesis because, although hatching asynchrony provides females with a means for increasing the male's contribution to parental care, females can only do so by also reducing their offspring's size and survival. Thus, females should be under selection to produce an intermediate hatching pattern that provides the best possible balance between the benefits of increased male parental effort and the costs due to reduced offspring fitness.

We found that mean larval mass was greater when parents provided care for longer. Previous work has found that parental care improves offspring growth, particularly during the early stages of larval development (Eggert *et al.*, 1998; Smiseth *et al.*, 2003). Thus, caring for the brood for longer has positive effects on the offspring's fitness. The fact that one of the parents often deserts the brood prematurely therefore suggests that there must be some cost of providing care, such as reduced lifespan or increased loss of body mass, or that there are some benefits of deserting the brood, such as increased opportunities for breeding (Royle *et al.*, 2012). If the sexual conflict hypothesis is to be supported, the benefits that the female gains from increasing the male's contribution towards care for highly asynchronous broods, thereby allowing the female to reduce her own costs of care, should outweigh the detrimental effect of reduced offspring fitness. Currently, it is unclear what costs parents incur from providing care in burying beetles. There does not appear to be an immediate physiological cost of breeding given that almost all parents in our study gained mass during breeding – a result that also has been found in the closely related *N. orbicollis* (Scott & Traniello, 1990) – and that parents that remained with the brood for longer gained proportionally more mass. Furthermore, we did not find any long-term cost of caring, as there were no effects of the hatching pattern or the duration of parental care on female lifespan. In contrast to what was found by Boncoraglio & Kilner (2012), we found no effect of male presence after hatching on female lifespan. This may reflect that the benefit to females of being assisted by a male depends on the ecological context. For example, male presence could be detrimental if there is competition for food between parents and offspring on small carcasses because dispersing brood mass is lower for broods reared by males that gain mass (Scott & Gladstein, 1993) and females sometimes even kill males on very small carcasses (Bartlett, 1988). The carcasses used in the present study were large enough to support 20 larvae, and thus, there were probably sufficient resources for both parents to feed without depriving the offspring of food.

Although our study did not identify a benefit to the female of reducing her duration of care, it is possible that we were unable to detect such a benefit in our laboratory experiment. For example, females may benefit from deserting earlier by reducing their risk infection by microorganisms present on the carcass. We always used fresh carcasses in our experiment, but females in the field may breed on carcasses that have begun to decompose before the start of the breeding attempt (Steiger *et al.*, 2011). Furthermore, females may benefit from deserting earlier by reducing the risk of injury or death during fights with conspecifics. We always excluded competitors in our experiments, while females in the field may face both interspecific and intraspecific competitors, which may cause injury or death. Thus, future work on the potential benefits to females from increasing the amount of male care should consider designs that mimic the harsher conditions these beetles face in the wild.

Our study is the first to test the sexual conflict hypothesis in a nonavian species. We found some support for the sexual conflict hypothesis in the burying beetle *N. vespilloides*. As predicted, we found that males and females responded differently to hatching patterns: males provided care for longer in highly asynchronous broods whereas the opposite was true of females. Our findings suggest that asynchronous hatching may play a role in the resolution of sexual conflict over parental care in *N. vespilloides*. However, we did not find any evidence that females benefitted from reducing their duration of care, and we found costs of high levels of hatching asynchrony in terms of reduced larval growth and survival. We argue that hatching asynchrony would only be a viable strategy for females to increase the male's contribution to care if the benefits to females from reducing their own costs of care outweigh the costs of reduced offspring fitness. We recommend that future studies on the sexual conflict hypothesis recog-

nize the importance of assessing fitness consequences for parents and offspring in addition to studying changes in each parent's contribution towards parental care.

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Original Article

# Asynchronous hatching in a nonavian species: a test of the hurry-up hypothesis

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The hurry-up hypothesis suggests that completing reproduction as soon as possible is favored when the quantity or quality of resources used for breeding declines over time. However, completing reproduction sooner may incur a cost if it leads to an asynchronous hatching pattern that reduces overall growth and survival of offspring. Here, we present the first test of the hurry-up hypothesis in a nonavian system, the burying beetle *Nicrophorus vespilloides*, which breeds on small vertebrate carcasses. To this end, we conducted 2 experiments in which we provided females with an incentive to complete reproduction sooner by giving them carcasses that varied either with respect to decomposition (resource quality) or size (resource quantity). We recorded the delay until laying and measures of the laying pattern and fitness consequences for the offspring. As predicted, we found that larvae dispersed from the carcass earlier when females commenced oviposition sooner and that laying spread was greater when females commenced egg laying earlier. However, we found no evidence that females commenced egg laying earlier on either decomposed or larger carcasses. Our results suggest that, although asynchronous hatching might emerge as a by-product of parents attempting to complete reproduction sooner, there is no evidence that females attempt to complete reproduction sooner under conditions where this would be favorable. Our results are therefore inconsistent with the hurry-up hypothesis.

*Key words*: **egg size, environmental conditions, *Nicrophorus vespilloides*, offspring fitness, resource quality, resource quantity.**

## INTRODUCTION

In species where parents provide food or other forms of care for their dependent offspring, parents may be under selection to respond to deteriorating environmental conditions by shortening the time from the onset of breeding until the offspring reach independence (Hussell 1972; Clark and Wilson 1981; Slagsvold 1986). This condition might be met when there is a decline in quantity or quality of food resources (Hussell 1972; Clark and Wilson 1981; Slagsvold 1986), an increase in predation rate on dependent offspring, or when climatic conditions worsen over time (Magrath 1990). The hurry-up hypothesis was proposed to explain how parent birds might respond to deteriorating environmental conditions (Hussell 1972; Clark and Wilson 1981) but the hypothesis may apply in general across animal taxa. Female birds are constrained from laying more than one egg each day, which means that it takes them several days to complete a clutch. However, because avian eggs only start developing once parents start incubating, parents control embryonic development by simply adjusting the onset of incubation (Clark and Wilson 1981). Thus, if parent birds perceive that the peak in food supply will occur earlier than anticipated at the start of laying, they can accelerate offspring development by commencing incubation earlier instead of waiting until the clutch has been completed. However, studies on birds show that parental attempts to shorten the time until offspring independence in response to dwindling food supplies towards the end of the breeding season are also associated with greater levels of asynchronous hatching (Gibb 1950; Van Balen 1973; Nisbet and Cohen 1975; Slagsvold 1982; Slagsvold 1986; Hébert and McNeil 1999).

The hurry-up hypothesis suggests that asynchronous hatching emerges as a nonadaptive by-product due to parent birds being under selection to commence incubation before the clutch has been completed (Clark and Wilson 1981). There is good evidence that hatching asynchrony can incur substantial fitness costs because it often leads to asymmetric sibling competition with a detrimental impact on the survival and/or growth of the last offspring to hatch (Lack 1947; Clark and Wilson 1981; Hillström and Olsson 1994; Smiseth et al. 2007). Thus, parents should be under selection to balance the benefits of shortening the time until the first offspring reach independence against the costs of producing an asynchronous hatching pattern that reduces the overall growth and survival of offspring. Furthermore, parents may be under selection to offset any undesirable fitness consequences of asymmetric sibling competition (Clark and Wilson 1981). For example, in several birds with asynchronous hatching, parents produce larger eggs toward the end of the laying sequence (Schrantz 1943; Kendeigh et al.

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1956; Holcomb 1969; Howe 1976; Bryant 1978; Howe 1978). This may compensate for some of the fitness costs of asymmetric sibling competition given that offspring hatching mass is positively correlated with egg size (Krist 2011) and chicks hatching from heavier eggs have higher early growth (Hillström 1999).

Although the hurry-up hypothesis was proposed for birds where parents incubate their eggs, it may also apply to nonavian species where eggs develop without incubation, provided that there is a trade-off between commencing oviposition soon after the initiation of reproduction and laying eggs synchronously. Burying beetles of the genus *Nicrophorus* are an ideal nonavian study system in which to test the hurry-up hypothesis because they exhibit hatching asynchrony similar to that of many altricial birds (Müller and Eggert 1990; Smiseth et al. 2006; Takata et al. 2015). These beetles breed on carcasses of small vertebrates, which are buried underground (Scott 1998). Females lay eggs in the surrounding soil (Pukowski 1933) and the hatching pattern is determined by the period of time over which the eggs are laid, termed "laying spread" (Smiseth et al. 2006; Takata et al. 2015). The degree of hatching asynchrony is highly variable in *N. vespilloides*, ranging from 16 to 56 h with a mean of 30 h, which is considerable relative to the duration of embryonic development (59 h) and the duration of parental food provisioning (72 h) (Smiseth et al. 2006). Burying beetles could potentially reduce the delay until the offspring reach independence by starting to lay eggs sooner after encountering the carcass. However, because females do not mature their oocytes until they secure a carcass for breeding (Wilson and Knollenberg 1984), starting to lay very soon after encountering the carcass may be costly as females may not have consumed enough resources to mature all of their oocytes before commencing oviposition. If this is the case, starting to lay soon after encountering the carcass may be associated with a greater laying spread and a greater level of hatching asynchrony if there is a trade-off between accelerating the maturation of some oocytes and maturing all oocytes synchronously. This may be undesirable due to increased mortality of the last hatched offspring in asynchronous broods (Smiseth et al. 2008; Takata et al. 2014; Ford and Smiseth 2016).

Here, we present the results of 2 experiments designed to test the hurry-up hypothesis in *N. vespilloides*. We provided female beetles with an incentive to shorten the time until offspring independence in response to variation in either resource quality or resource quantity. We used carcass decomposition as a proxy for resource quality while we used carcass size as a proxy for resource quantity. We predicted that females would shorten the time until offspring independence when breeding on decomposed carcasses because the value of the carcass should decrease over time due to an increase in microbial load. Indeed, carcass decomposition has a detrimental effect on larval growth and survival (Rozen et al. 2008) and egg survival (Jacobs et al. 2014). Meanwhile, we predicted that females would start to lay sooner when breeding on larger carcasses because larger carcasses are more difficult to roll into a ball; so, it is more difficult to control microbial activity due to the larger surface area remaining exposed (Trumbo 1992). Furthermore, it is more difficult for the parents to keep the carcass coated with antimicrobial secretions, which may provide an incentive to complete reproduction sooner on larger carcasses, before decomposition of the carcass becomes detrimental to the larvae. In addition, larger carcasses are more likely to be usurped by other burying beetles (Trumbo 1991), which will kill the entire resident brood (Trumbo 1990). Completing reproduction as soon as possible is particularly important on large carcasses because the risk of usurpation is not constant; in *N. orbicollis* the risk of takeovers decreases after 5 days (Robertson 1993), possibly because depletion of the carcass due to larvae feeding makes it more difficult for free-flying beetles to detect. Moreover, the brood is less likely to be killed by a vertebrate scavenging the carcass if reproduction is completed sooner (Trumbo 1992). Here, we addressed the following 4 questions: 1) Are the assumptions of the hurry-up hypothesis met in this system? If so, females could shorten the time to offspring independence by commencing oviposition sooner and there would be a trade-off between commencing oviposition sooner and laying the eggs more synchronously. 2) Do females adjust egg laying in response to variation in environmental conditions? We predicted that females breeding on decomposed or larger carcasses would shorten the time until offspring independence by commencing oviposition sooner and that they would lay their eggs more asynchronously. 3) Is breeding success influenced by females commencing oviposition sooner and by patterns of egg laying? We predicted that starting to lay sooner would be beneficial and that asynchrony would be detrimental in terms of the size, number or survival of larvae 4) Do females compensate for the fitness costs of asymmetric sibling competition? If so, we predicted that females would lay larger eggs towards the end of the laying sequence.

## METHODS

### Study animals

The beetles used in this study were from an outbred laboratory population maintained at the University of Edinburgh. Beetles were housed individually in clear plastic boxes (124 mm × 82 mm × 22 mm). They were kept at 20 ± 2 °C (mean ± range) under constant light and were fed small pieces of organic beef twice a week. The beetles were sexually mature and of prime reproductive age (18–26 days posteclosion) at the start of the experiments.

### Experimental procedures

The aim of the carcass decomposition experiment was to determine whether females attempt to shorten the time until offspring independence when the carcass is at a more advanced stage of decomposition at the start of the breeding attempt and whether this is associated with greater levels of hatching asynchrony. We provided females with either a fresh mouse carcass that had been thawed immediately before it was provided to the beetles ($n$ = 36) or a decomposed carcass that had been left in the breeding box for 10 days before it was provided to the beetles ($n$ = 35). The carcasses used in this experiment weighed 23.32–29.50 g (mean 25.57 g) when initially thawed. We initially set up pairs on 42 fresh and 59 decomposed carcasses but excluded all pairs where the eggs did not hatch (fresh carcasses: $n$ = 3; decomposed carcasses: $n$ = 21) and where there were technical problems (fresh carcasses: $n$ = 3; decomposed carcasses: $n$ = 3). The aim of the carcass size experiment was to determine whether females attempt to complete reproduction sooner on larger carcasses. We allowed beetles to breed on a range of carcasses ($n$ = 82) from 4–27 g, which is within the range of vertebrate carcasses utilized by beetles in the wild (range: 1–37 g; Müller et al. 1990). We initially set up 92 pairs but excluded 1 pair that failed to lay eggs and 9 pairs that failed to hatch larvae.

For both experiments, we paired unrelated virgin males and females and placed them in a clear breeding box (170 mm × 120 mm × 60 mm) containing <1 cm of moist compost. We supplied each pair with a previously frozen mouse carcass (supplied from Livefoods

Direct Ltd, Sheffield, UK). Eggs are visible at the bottom of the breeding box and can be seen on images obtained by placing the boxes on flat-bed scanners (Canon Canoscan 9000F Mark II, Canon Inc., Tokyo, Japan) (Ford and Smiseth 2016). In the small amount of soil used, the visible number of eggs is very similar to the actual clutch size (Monteith et al. 2012). We scanned the breeding boxes every hour using Vuescan professional edition software (Hamrick Software, Sunny Isles Beach, FL) until the eggs hatched. In accordance with previous studies, we defined a clutch as comprised of all eggs laid before the first larva hatched (Müller 1987; Steiger 2013). From the scanned images, we counted the number of new eggs laid each hour to determine the laying spread (the time between the first and last egg being laid) and the clutch size (the number of eggs laid before the first egg hatched, Müller et al. 1990). We excluded clutches where all eggs failed to hatch because females will continue to lay eggs if larvae do not arrive at the carcass, resulting in aberrant laying patterns (Müller 1987). We also calculated a laying skew index reflecting the extent to which laying is skewed toward the earlier part of the laying period, using the formula $\Sigma((t_i-t_m)/t_m)p_i$, where $p_i$ is the proportion of the total clutch laid each hour, $t_i$ is the time interval starting from the initiation of oviposition and $t_m$ is the middle of the laying period (Smiseth et al. 2008). We recorded the number of days since pairing until the larvae dispersed from the carcass and counted the number of larvae dispersing from each brood. At the time of dispersal, we also weighed the entire brood mass and calculated the average larval mass by dividing the brood mass by the number of larvae in the brood.

We measured egg size in the carcass decomposition experiment to determine whether females compensated for the consequences of asymmetric sibling competition caused by asynchronous hatching by increasing egg size across the laying sequence. Using ImageJ (Abramoff et al. 2004), we split the clutch approximately into thirds and measured the length and width of 3 eggs from the first third (first eggs), 3 eggs from the second third (middle eggs), and 3 eggs from the last third of the clutch (last eggs). We only measured eggs that were lying flat against the bottom of the box. If there were several suitable eggs on a single scan, we randomly chose which ones to measure. Three broods were excluded for the middle eggs because there were no suitable eggs to measure that had not already been measured as either first or last eggs due to small clutch sizes. Eggs were measured from the second image on which they were present as they appear to shrink during the first hour after being laid. Each egg was measured 3 times and the mean length and width for each egg was used to calculate a prolate spheroid volume (V) using the equation $V = (1/6)\pi w^2 L$, where w is the width and L the length of the egg (Berrigan 1991). We did not measure eggs in the carcass size experiment because laying asynchrony did not differ with carcass size once the greater clutch sizes on larger carcasses were taken into account and therefore we did not expect that there would be sufficient asymmetric sibling competition to elicit a response from females attempting to compensate.

### Statistical analyses

We carried out the statistical analyses in R (R Core Team 2014). We constructed generalized linear models. Graphical model validation showed that the residuals of the model were normally distributed and homoscedastic, that there was no over dispersion and that the model was not biased by unduly influential observations. Variance inflation factors confirmed that there was not excessive collinearity of the variables. We carried out model refinement through backwards stepwise deletion to determine the significance of each term comparing otherwise identical models either including or excluding the term of interest using likelihood ratio tests. We then removed the least significant term and repeated the process until only significant terms remained. We used correlation tests (Pearson's correlation test, or Kendall's Tau correlation where the data did not fit a normal distribution) to investigate whether a shorter delay until laying after females encountered the carcass was associated with earlier larval dispersal or greater laying asynchrony.

We initially produced a model for each response variable containing only treatment to determine whether there was an effect of carcass decomposition or carcass size. We then produced full models containing covariates to investigate whether any difference between treatments was solely due to variation between treatments of other correlated variables. All analyses for the carcass decomposition experiment contained treatment (fresh or decomposed carcass) and all analyses for the carcass size experiment contained treatment (carcass size), as these were the focus of the experimental manipulation (Tables 1 and 2). The models for laying skew and laying spread included clutch size because it may take longer to lay larger clutches. We also included the delay until the first egg was laid in the models for laying skew because starting to lay sooner after encountering the carcass might affect laying patterns. In the models for the time until dispersal, we included laying spread because larvae in synchronous broods disperse sooner after arriving at the carcass than those in asynchronous broods (Ford and Smiseth 2016) and clutch size because larger clutches may use up the resource more quickly and therefore disperse sooner. The models for the absolute number of larvae dispersing and the proportion of eggs producing larvae that survived to dispersal included laying spread and the delay until the first egg was laid to test for a cost of asynchronous laying or starting to lay sooner after encountering the carcass. We also included clutch size in the models for the number of larvae dispersing because the number of dispersing larvae is limited by the number of eggs. We included clutch size in the models for the proportion of eggs producing larvae that survived to dispersal because females usually lay a greater number of eggs than the number of larvae the carcass can support so in large clutches a smaller proportion of the clutch may go on to produce dispersing larvae even if the viability of the eggs does not differ. The models for the mean larval mass at dispersal (excluding broods for which larvae hatched but no larvae survived to dispersal) included laying spread and the delay until the first egg was laid to test for a cost of asynchronous hatching or starting to lay sooner and the number of larvae dispersing in case there was a trade-off between number and size of larvae (Smiseth et al. 2014).

To determine whether egg size varied with laying order in the carcass decomposition experiment, we constructed a general linear mixed model using the restricted maximum likelihood method including laying spread, delay until the first egg was laid, stage (first, middle, or last eggs) and treatment (fresh or decomposed carcasses) and the interaction between stage and treatment as fixed effects and brood as a random effect in the lme4 package (Bates et al. 2015). We carried out stepwise model reduction using likelihood ratio tests.

## RESULTS

### Are the assumptions of the hurry-up hypothesis met?

We found that larvae dispersed from the carcass earlier when females commenced oviposition sooner in the carcass decomposition

**Table 1**

**Outputs of models investigating female laying behavior in response to carcass decomposition (treatment) and the consequences for the offspring**

| Response | Model terms | Test statistic | *P*-value | Parameter estimate, SE | 95% confidence intervals for parameter estimates | Error family |
|---|---|---|---|---|---|---|
| Time until first egg | Treatment | $F_{1,69} = 3.04$ | 0.0857 | −0.015, 0.008 | −0.030, 0.0005 | Gamma |
| Time until hatching | Treatment | $F_{1,69} = 0.45$ | 0.505 | −0.0003, 0.0004 | −0.030, 0.0005 | Inverse gaussian |
| Time until dispersal | Treatment | $F_{1,61} = 0.40$ | 0.528 | 0.139, 0.219 | −0.290, 0.567 | Gaussian |
| | Clutch size | $F_{1,60} = 5.92$ | **0.018** | −0.029, 0.012 | −0.053, −0.006 | |
| | Laying spread | $F_{1,59} = 0.07$ | 0.798 | 0.003, 0.011 | −0.019, 0.025 | |
| Laying spread | Treatment | $F_{1,69} = 26.54$ | **<0.0001** | −0.0009, 0.0002 | −0.001, −0.0006 | Inverse gaussian |
| | Clutch size | $F_{1,68} = 2.73$ | 0.103 | <−0.0001, <0.0001 | <−0.0001, <0.0001 | |
| Laying skew | Treatment | $F_{1,69} = 0.03$ | 0.870 | −0.007, 0.040 | −0.085, 0.072 | Gaussian |
| | Clutch size | $F_{1,68} = 0.04$ | 0.837 | −0.0005, 0.002 | −0.005, 0.004 | |
| | Delay until first egg | $F_{1,68} = 1.39$ | 0.242 | −0.004, 0.004 | −0.011, 0.003 | |
| Proportion of eggs surviving to dispersal | Treatment | $F_{1,69} = 8.92$ | **0.004** | 0.209, 0.070 | 0.072, 0.346 | Gaussian |
| | Laying spread | $F_{1,68} = 6.07$ | **0.016** | −0.009, 0.004 | −0.016, −0.002 | |
| | Clutch size | $F_{1,67} = 2.23$ | 0.140 | −0.006, 0.004 | −0.014, 0.002 | |
| | Delay until first egg | $F_{1,67} = 1.97$ | 0.165 | 0.009, 0.006 | −0.003, 0.021 | |
| Number of larvae at dispersal | Treatment | $X^2_{1,69} = 17.64$ | **<0.0001** | 0.936, 0.220 | 0.504, 1.368 | Negative binomial |
| | Clutch size | $X^2_{1,68} = 0.72$ | 0.397 | 0.010, 0.013 | −0.013, 0.034 | |
| | Laying spread | $X^2_{1,68} = 1.41$ | 0.235 | −0.013, 0.012 | −0.035, 0.009 | |
| | Delay until first egg | $X^2_{1,68} = 0.05$ | 0.816 | 0.004,0.020 | −0.032, 0.042 | |
| Mean larval mass (excluding zeroes) | Treatment | $F_{1,61} = 99.78$ | **<0.0001** | 0.078, 0.008 | 0.063, 0.094 | Gaussian |
| | Number of larvae | $F_{1,60} = 0.93$ | 0.339 | −0.0003, 0.0003 | −0.001, 0.0003 | |
| | Laying spread | $F_{1,60} = 1.63$ | 0.207 | 0.001, 0.0004 | −0.0003, 0.001 | |
| | Delay until first egg | $F_{1,60} = 0.004$ | 0.951 | <−0.0001, 0.0007 | −0.001, 0.001 | |
| Mean larval mass (including zeroes) | Treatment | $F_{1,69} = 99.55$ | **<0.0001** | 0.103, 0.010 | 0.083, 0.124 | Gaussian |
| | Number of larvae | $F_{1,68} = 1.21$ | 0.275 | 0.001, 0.0005 | −0.0004, 0.001 | |
| | Laying spread | $F_{1,68} = 0.06$ | 0.804 | −0.0001, 0.0006 | −0.001, 0.001 | |
| | Delay until first egg | $F_{1,68} = 0.55$ | 0.462 | −0.0007, 0.001 | −0.003, 0.001 | |

Significant effects are shown in bold.

experiment (Pearson's correlation $t_{61} = 3.99$, $P = 0.0002$; Figure 1a). However, there was no evidence that larvae dispersed from the carcass earlier when females commenced oviposition sooner in the carcass size experiment (Pearson's correlation $t_{67} = 0.055$, $P = 0.956$; Figure 1b). Thus, we found some evidence that females can shorten the time to offspring independence by commencing oviposition sooner but this response was conditional upon the specific environmental conditions of the 2 experiments.

As expected if starting to lay sooner after encountering the carcass leads to greater laying asynchrony, we found that laying spread was greater when females commenced egg laying earlier in the carcass size experiment (Pearson's correlation $t_{80} = -2.27$, $P = 0.026$; Figure 1d) and for females breeding on fresh carcasses in the carcass decomposition experiment (Kendall's Tau correlation $z = -2.15$, $P = 0.032$) However, there was no evidence for such a trade-off for females breeding on decomposed carcasses ($z = -0.17$, $P = 0.863$; Figure 1c). Thus, we found some evidence for a trade-off between starting to lay earlier and laying synchronously but only when females bred on fresh carcasses.

## Do females adjust egg laying in response to environmental conditions?

Contrary to what we predicted, there was no evidence that females commenced oviposition sooner on decomposed carcasses compared to fresh ones ($F_{1,69} = 3.04$, $P = 0.086$; Figure 2a) or as carcass size increased ($F_{1,80} = 2.19$, $P = 0.142$; Figure 2b). As a consequence, there was no difference in either the time until the first larvae hatched ($F_{1,69} = 0.45$, $P = 0.505$) or the time until larval dispersal between females breeding on decomposed and fresh carcasses ($F_{1,62} = 0.40$, $P = 0.528$). Likewise, there was no difference in the time until hatching started between females breeding on different sized carcasses ($F_{1,79} = 1.84$, $P = 0.179$). We found that larval dispersal was delayed as a function of increasing carcass size ($F_{1,67} = 8.66$, $P = 0.004$).

In contrast to what we predicted, we found that laying spread was less pronounced on decomposed carcasses than on fresh carcasses ($F_{1,69} = 26.54$, $P < 0.0001$; Figure 2c). This difference was independent of clutch size as it persisted when we controlled for the effect of clutch size ($F_{1,68} = 13.49$, $P = 0.00047$). Thus, females were able to lay a given clutch size more synchronously on decomposed carcasses. There was a significant increase in laying spread with carcass size ($F_{1,80} = 6.22$, $P = 0.015$; Figure 2d). However, this effect was not statistically significant when we controlled for the effects of clutch size ($F_{1,79} = 0.28$, $P = 0.597$). We included clutch size in the model because females laid a greater number of eggs on larger carcasses (Pearson's correlation: $t = 6.13$, $P < 0.0001$) and females took longer to lay larger clutches ($F_{1,79} = 8.34$, $P = 0.005$) on a given carcass size. Thus, the increase in laying spread on larger carcasses was likely to be solely due to the concurrent increase in clutch sizes. The laying skew index was negative for more than 90% of broods in both experiments, reflecting that the majority of the eggs were laid toward the start of the laying period. The laying skew index did

**Table 2**
**Outputs of models investigating female laying behavior in response to carcass size and the consequences for the offspring**

| Response | Model terms | Test statistic | *P*-value | Parameter estimate, SE | 95% confidence intervals for parameter estimates | Error family |
|---|---|---|---|---|---|---|
| Time until first egg | Carcass mass | $F_{1,80} = 2.19$ | 0.142 | 0.001, 0.0007 | −0.0004, 0,002 | Gamma |
| Time until hatching | Carcass mass | $F_{1,79} = 1.84$ | 0.179 | <−0.0001,<0.0001 | <−0.0001,<0.0001 | Inverse gaussian |
| Time until dispersal | Carcass mass | $F_{1,67} = 8.66$ | **0.004** | 0.040, 0.014 | 0.013,0.067 | Gaussian |
| | Clutch size | $F_{1,66} = 0.68$ | 0.411 | 0.011, 0.013 | −0.014,0.036 | |
| | Laying spread | $F_{1,66} = 1.03$ | 0.314 | 0.012, 0.011 | −0.011, 0.034 | |
| Laying spread | Carcass mass | $F_{1,80} = 6.22$ | **0.015** | <−0.0001,<0.0001 | <−0.0001,< −0.0001 | Inverse gaussian |
| | Clutch size | $F_{1,79} = 8.34$ | **0.005** | <−0.0001,<0.0001 | <−0.0001,< −0.0001 | |
| Laying skew | Carcass mass | $F_{1,80} = 3.38$ | 0.070 | −0.005, 0.003 | −0.010, 0.0003 | Gaussian |
| | Clutch size | $F_{1,79} = 1.52$ | 0.221 | −0.003, 0.003 | −0.008, 0.002 | |
| | Delay until first egg | $F_{1,79} = 0.17$ | 0.678 | 0.002, 0.004 | −0.006, 0.010 | |
| Proportion of eggs surviving to dispersal | Carcass mass | $F_{1,80} = 0.86$ | 0.356 | 0.004, 0.004 | −0.004, 0.012 | Gaussian |
| | Clutch size | $F_{1,79} = 0.007$ | 0.932 | 0.0003, 0.004 | −0.008, 0.008 | |
| | Laying spread | $F_{1,79} = 2.14$ | 0.148 | −0.005, 0.004 | −0.012, 0.002 | |
| | Delay until first egg | $F_{1,79} = 1.43$ | 0.235 | −0.007, 0.006 | −0.020, 0.005 | |
| Number of larvae at dispersal | Carcass mass | $X^2_{1,80} = 3.94$ | **0.047** | 0.031, 0.015 | 0.0004, 0.062 | Negative binomial |
| | Clutch size | $X^2_{1,79} = 3.81$ | .051 | 0.025, 0.014 | −0.0001,0.050 | |
| | Laying spread | $X^2_{1,79} = 0.17$ | 0.682 | −0.005, 0.012 | −0.029, 0.020 | |
| | Delay until first egg | $X^2_{1,79} = 2.89$ | 0.089 | −0.051, 0.023 | −0.108, 0.008 | |
| Mean larval mass (excluding zeroes) | Carcass mass | $F_{1,67} = 40.05$ | **<0.0001** | 0.004, 0.0006 | 0.003, 0.005 | Gaussian |
| | Number of larvae | $F_{1,66} = 22.48$ | **<0.0001** | −0.002, 0.0004 | −0.002, −0.001 | |
| | Laying spread | $F_{1,65} = 1.32$ | 0.255 | −0.0005, 0.0004 | −0.001, 0.0003 | |
| | Delay until first egg | $F_{1,65} = 0.91$ | 0.345 | 0.001, 0.001 | −0.001, 0.003 | |
| Mean larval mass (including zeroes) | Carcass mass | $F_{1,80} = 15.53$ | **0.0002** | 0.004, 0.001 | 0.002, 0.006 | Gaussian |
| | Number of larvae | $F_{1,79} = 9.55$ | **0.003** | 0.002, 0.0007 | 0.0008, 0.003 | |
| | Laying spread | $F_{1,78} = 0.03$ | 0.863 | 0.0002, 0.0009 | −0.002, 0.002 | |
| | Delay until first egg | $F_{1,78} = 0.30$ | 0.588 | −0.0008, 0.002 | −0.004, 0.002 | |

Significant effects are shown in bold.

not differ between females breeding on fresh and decomposed carcasses ($F_{1,69} = 0.03$, $P = 0.870$) and was not influenced by carcass size ($F_{1,80} = 3.38$, $P = 0.070$).

## Does ovipositing sooner and laying asynchronously influence breeding success?

There was no evidence that starting to lay sooner had any effects on breeding success as the time until females started laying had no effect on either the number of dispersing larvae (decomposition experiment $X^2_{1,68} = 0.05$, $P = 0.816$; carcass mass experiment $X^2_{1,79} = 2.89$, $P = 0.089$) or the mass of these larvae (carcass decomposition experiment $F_{1,60} = 0.0038$, $P = 0.951$; carcass size experiment $F_{1,65} = 0.91$, $P = 0.345$). Not only was the proportion of eggs hatching into larvae that reached independence lower on decomposed carcasses than fresh carcasses ($F_{1,69} = 8.92$, $P = 0.004$), there was also a detrimental effect of laying asynchrony on offspring survival with fewer offspring surviving in clutches with a greater laying spread ($F_{1,68} = 6.07$, $P = 0.016$). However, there was no effect of laying asynchrony on offspring survival in the carcass size experiment ($F_{1,79} = 2.14$, $P = 0.148$). Laying spread had no effect on the absolute number of larvae dispersing (carcass decomposition experiment $X^2_{1,68} = 1.413$, $P = 0.235$; carcass size experiment $X^2_{1,79} = 0.17$, $P = 0.682$), the time until dispersal (carcass decomposition experiment $F_{159} = 0.07$, $P = 0.798$; carcass size experiment $F_{1,66} = 1.03$, $P = 0.314$) or larval mass (carcass decomposition experiment $F_{1,60} = 1.63$, $P = 0.207$; carcass size experiment $F_{1,65} = 1.32$, $P = 0.255$).

## Do females lay larger eggs towards the end of the laying sequence?

Egg volume varied across the laying sequence ($X^2_2 = 86.24$, $P < 0.0001$), reflecting that last-laid eggs were smaller than first-laid eggs for females breeding on both decomposed (Wilcoxon matched pairs test: $V = 602$, $P < 0.0001$) and fresh carcasses ($V = 615$, $P < 0.0001$). Furthermore, egg volume was greater for females breeding on fresh carcasses than on decomposed ones ($X^2_1 = 5.60$, $P = 0.018$) and there was a significant effect of the interaction between carcass decomposition and laying order on egg volume ($X^2_2 = 8.00$, $P = 0.018$). This interaction effect reflected that there was a greater change in egg volume across the laying order for females breeding on fresh carcasses than for females breeding on decomposed carcasses. There was no evidence that egg volume was associated with either the time until females commenced oviposition ($X^2_1 = 0.01$, $P = 0.945$) or laying spread ($X^2_1 = 1.34$, $P = 0.250$).

# DISCUSSION

Here, we report the results of the first test of the hurry-up hypothesis in a nonavian species. This hypothesis was originally proposed as an explanation for asynchronous hatching in birds but would also apply to nonavian species, such as *N. vespilloides*, provided that the following assumptions are met: 1) starting to lay sooner shortens the time to offspring independence and 2) starting to lay sooner leads to greater laying asynchrony. Our study provides some evidence that both assumptions are met in *N. vespilloides*. First, females were able to shorten the time to offspring independence as larvae

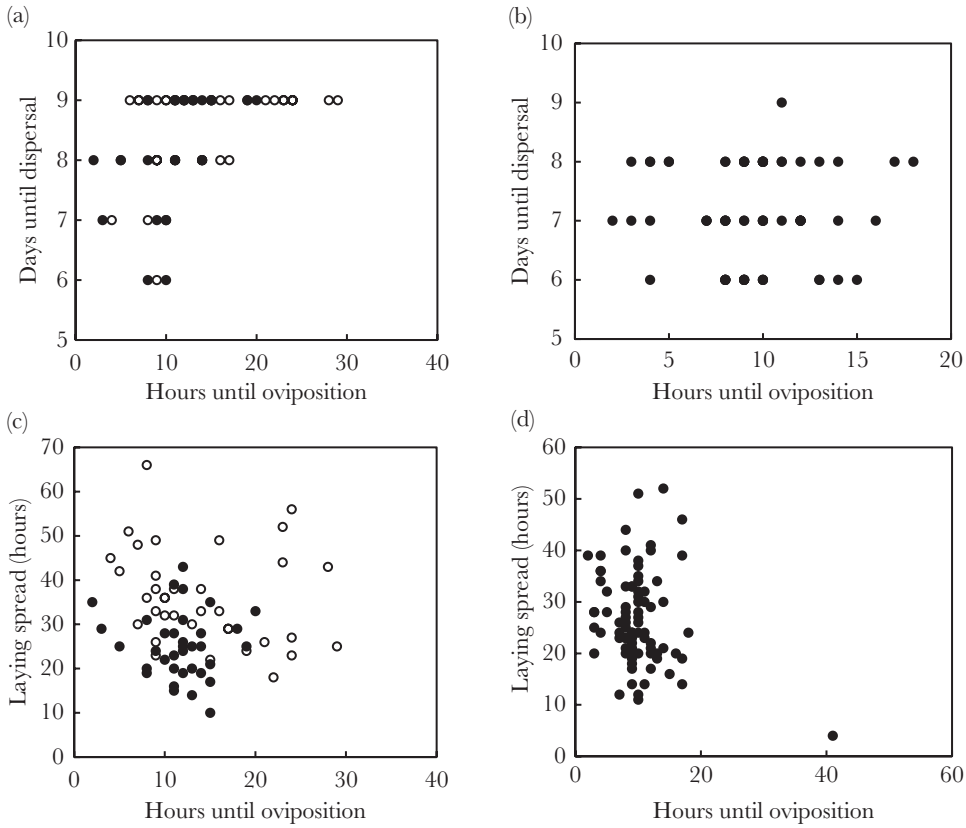


**Figure 1**
Effect of the delay until females commenced oviposition after encountering the carcass on the number of days until larval dispersal in the carcass decomposition experiment (a) and carcass size experiment (b) and on the laying spread (the number of hours between the first end last egg being laid) in the carcass decomposition experiment (c) and carcass size experiment (d). In (a) and (c), open circles represent values for females breeding on fresh carcasses and filled circles represent values for females breeding on decomposed carcasses.

dispersed earlier when females commenced oviposition sooner after encountering the carcass (though this was the case only in the carcass decomposition experiment). Second, there was evidence of a trade-off between commencing oviposition sooner and laying the eggs more synchronously (though this was not the case for females breeding on decomposed carcasses). Therefore, our results show that, as suggested by the hurry-up hypothesis, female burying beetles could shorten the time until offspring independence by starting to lay sooner after encountering the carcass, and that this in turn could lead to greater levels of laying asynchrony as a by-product. In addition to the 2 assumptions mentioned above, the following 3 predictions must also be supported for the hurry-up hypothesis to be accepted: 1) females should commence oviposition sooner when environmental conditions provide an incentive for accelerated offspring independence (such as breeding on decomposed or larger carcasses), 2) commencing oviposition sooner should have positive effects on breeding success, and 3) increased levels of hatching asynchrony should be detrimental for offspring fitness. Although multiple tests are required to test the hurry-up hypothesis, it is highly unlikely that it would be accepted due to Type 1 error given that this would require false positives for both assumptions as well as for all 3 predictions. As detailed below, we did not find sufficient evidence in support of the predictions of the hurry-up hypothesis in *N. vespilloides*. Furthermore, we urge caution when interpreting results for the covariates included in our models due to the potential for Type 1 errors associated with multiple testing.

In contrast to what we predicted, we found that females started ovipositing at a similar time after encountering the carcass regardless of whether they were breeding on decomposed or fresh carcasses. Consequently, there was no difference in the timing of larval dispersal between females breeding on decomposed and fresh carcasses. The latter result contrasts with a previous study on the same species, which found that dispersal occurred later on decomposed carcasses than on fresh carcasses (Rozen et al. 2008). The different results of this previous study may reflect differences in methodology as it used experimental foster broods that were completely synchronous and smaller than our natural broods (Rozen et al. 2008), while we allowed females to rear their own broods without interference. We also found that females started laying at the same time after encountering a carcass regardless of its size. This result contrasts with previous work in the closely related *N. orbicollis*, which showed that larger carcasses took longer to bury and prepare and that oviposition therefore was delayed on these carcasses (Scott and Gladstein 1993; Scott and Panaitof 2004). This discrepancy may be due to the differences in carcass preparation and burial between the 2 species, reflecting that *N. orbicollis* need to bury the carcass fully beneath the soil, whereas *N. vespilloides* does not (Pukowski 1933). We found that larvae dispersed later on larger carcasses, possibly reflecting that larvae on smaller carcasses exhausted the resource more quickly. This is consistent with previous work on *N. orbicollis*, which found that larvae on larger carcasses took longer to develop leading to later dispersal (Trumbo 1991; Scott and Gladstein 1993).

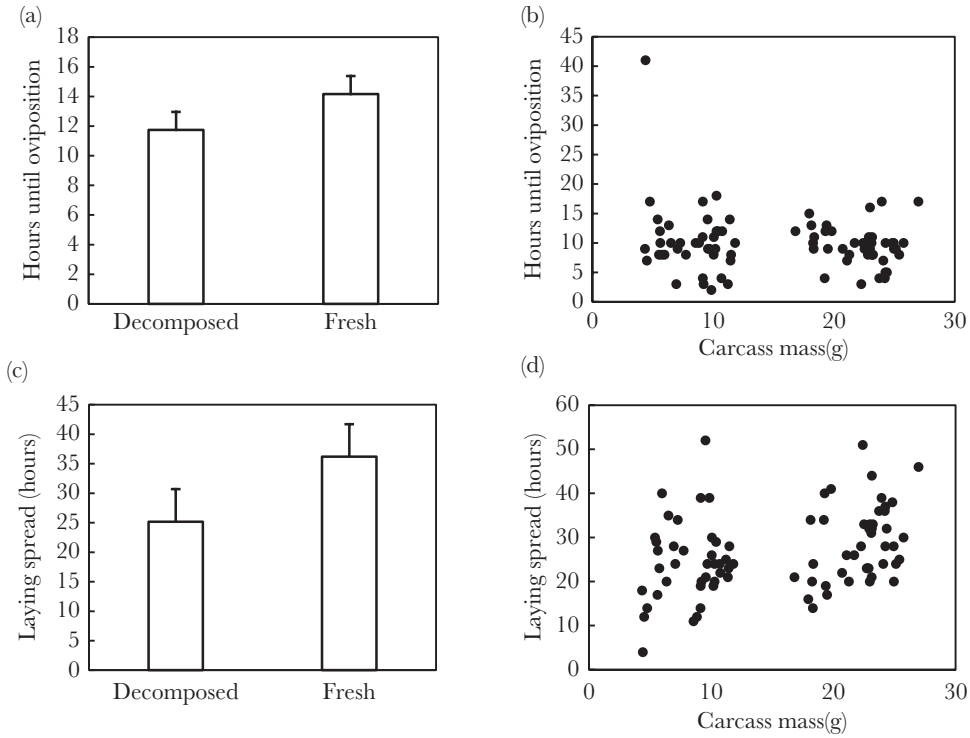


**Figure 2**
Effect of carcass decomposition (a) and carcass size (b) on the delay until females commenced oviposition after encountering the carcass and effect of carcass decomposition (c) and carcass size (d) on the laying spread (the number of hours between the first end last egg being laid). Error bars show standard errors.

Although there may be an incentive to accelerate offspring independence on larger carcasses due the increased risk of usurpation by other beetles and increased difficulty controlling microbial growth, larger carcasses also represent a more valuable resource that can sustain a greater number of larvae and larger larvae than smaller carcasses. These factors could interact in a complex manner to determine the optimal laying behavior depending on the size of the carcass being used. In summary, our results provide no evidence that females shortened the time until the offspring reached independence on decomposed or larger carcasses by commencing oviposition sooner on these carcasses.

We found that there was a greater laying spread on larger carcasses but only because females laid a greater number of eggs on these carcasses and females took longer to complete larger clutches. This finding suggests that females adjust clutch size rather than hatching pattern in response to variation in carcass size and that any effect on laying spread is a by-product of the number of eggs laid. We found that females breeding on decomposed carcasses laid a given clutch size more synchronously than those breeding on fresh carcasses, suggesting that the former may attempt to lay as synchronously as possible without incurring too great a cost through a decrease in egg size or the female's body condition. We found some evidence of a cost of asynchronous laying in that fewer offspring survived from the egg stage until dispersal in more asynchronous broods in the carcass decomposition experiment. Previous work suggests that larvae hatching from the last-laid eggs in asynchronous clutches suffer increased mortality (Smiseth et al. 2008; Takata et al. 2014; Ford and Smiseth 2016). When females breed on decomposed carcasses, late-laid eggs may be at a greater disadvantage due to the poor value of the carcass as a nutritional resource and reduced egg survival caused by microbes (Jacobs et al. 2014). Thus, under these circumstances, it may be highly advantageous for females to minimize laying spread. In contrast, it may be less advantageous for females to minimize laying spread when breeding on fresh carcasses, where moderate hatching asynchrony has a negligible effect on size or number of offspring. We found no evidence of a cost of asynchronous hatching for any aspect of offspring fitness in the carcass size experiment. The detrimental effects of hatching asynchrony may be less pronounced on larger carcasses (Müller et al. 1990) because there are sufficient resources for later-hatched larvae to survive even if they are considerably smaller than their siblings. This may explain why females breeding on large carcasses do not appear to attempt to minimize laying spread in large clutches because moderate hatching asynchrony is not detrimental under these circumstances.

If females attempted to compensate for competitive asymmetries due to asynchronous hatching, we might expect an increase in egg size across the laying sequence, as reported for some birds (Schrantz 1943; Kendeigh et al. 1956; Holcomb 1969; Howe 1976; Bryant 1978; Howe 1978; Hillström 1999). Although egg size did vary with laying order, we found that egg volume decreased from first to last eggs for both fresh and decomposed carcasses, which is in the opposite direction to what we predicted. The decrease in egg size across the laying order would further disadvantage the last larvae to hatch because smaller eggs have lower survival as shown in *N. quadripunctatus* (Takata et al. 2015). The last offspring to hatch are out-competed by their siblings that hatched earlier and have already grown to a larger size (Smiseth et al. 2007). However, due to the lesser laying spread on decomposed carcasses, competitive asymmetries would be reduced relative to fresh carcasses because the earlier larvae will have had less of a head start. Laying the clutch as synchronously as possible

when breeding on a decomposed carcass may therefore reduce competitive asymmetries to a greater extent than adjusting egg size. Thus, females may favor completion of laying as soon as possible over increasing egg volume across the clutch as a means to compensate for competitive asymmetries due to asynchronous hatching.

Overall, our results are not consistent with the hurry-up hypothesis given that females do not attempt to accelerate offspring independence under conditions where this would be favorable, such as when breeding on large carcasses or carcasses that have already started to decompose. Given that females breeding on decomposed carcasses laid more synchronously than those breeding on fresh carcasses, our results also show that females detect cues about the state of the carcass and adjust their laying spread accordingly. Our results also show that there is a survival cost to offspring in asynchronous clutches in the carcass decomposition experiment while there are no detectable benefits of asynchronous laying in any treatment. It is therefore unclear why hatching asynchrony occurs given that females are capable of laying more synchronously. Asynchronous hatching has evolved independently in many different taxa including insects, reptiles, fish, and birds. However, no consensus has yet been reached on how asynchronous hatching evolves in any system. Several hypotheses have been proposed to explain the occurrence of asynchronous hatching in birds, many of which are based on constraints on the onset of incubation or adaptive incubation patterns. These hypotheses do not apply to *N. vespilloides* because it does not incubate its eggs. Previous work on *N. vespilloides* has found no evidence for the peak load reduction (Smiseth and Morgan 2009) and sexual conflict hypotheses (Ford and Smiseth 2016). Thus, the only remaining hypotheses are the brood reduction (Lack 1947; Lack 1954) and insurance hypotheses (Stinson 1979). It seems unlikely that the original version of the brood reduction hypothesis (Lack 1947, 1954) would apply to *N. vespilloides* because brood reduction in this species occurs through filial cannibalism rather than sibling competition (Bartlett 1987). Nevertheless, late-hatched larvae grow less well and beg more (Smiseth et al. 2008), and are at a higher risk of being the victim of filial cannibalism (Andrews and Smiseth 2013; Takata et al. 2013). Thus, a modified version of this hypothesis may apply to burying beetles if asynchronous hatching somehow facilitates brood reduction through filial cannibalism. The insurance hypothesis (Stinson 1979) suggests asynchronous hatching serves as insurance against mortality of core offspring. This hypothesis may apply to burying beetles because many eggs fail to hatch and females may produce additional eggs as insurance (Bartlett 1987). The hypothesis proposes that late-hatched marginal offspring normally only survive if core offspring fail to hatch or die soon after hatching. The brood reduction hypothesis and insurance hypothesis may interact because, if all core offspring hatch, asynchronous hatching may provide an efficient mechanism for brood reduction (Forbes 1990). Further studies on asynchronous hatching in our system and other nonavian systems should therefore focus on these hypotheses.

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Data accessibility: Analyses reported in this article can be reproduced using the data provided by Ford and Smiseth (2017).

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# Differential effects of offspring and maternal inbreeding on egg laying and offspring performance in the burying beetle *Nicrophorus vespilloides*

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## Abstract

We investigate the effect of offspring and maternal inbreeding on maternal and offspring traits associated with early offspring fitness in the burying beetle *Nicrophorus vespilloides*. We conducted two experiments. In the first experiment, we manipulated maternal inbreeding only (keeping offspring outbred) by generating mothers that were outbred, moderately inbred or highly inbred. Meanwhile, in the second experiment, we manipulated offspring inbreeding only (keeping females outbred) by generating offspring that were outbred, moderately inbred or highly inbred. In both experiments, we monitored subsequent effects on breeding success (number of larvae), maternal traits (clutch size, delay until laying, laying skew, laying spread and egg size) and offspring traits (hatching success, larval survival, duration of larval development and average larval mass). Maternal inbreeding reduced breeding success, and this effect was mediated through lower hatching success and greater larval mortality. Furthermore, inbred mothers produced clutches where egg laying was less skewed towards the early part of laying than outbred females. This reduction in the skew in egg laying is beneficial for larval survival, suggesting that inbred females adjusted their laying patterns facultatively, thereby partially compensating for the detrimental effects of maternal inbreeding on offspring. Finally, we found evidence of a nonlinear effect of offspring inbreeding coefficient on number of larvae dispersing. Offspring inbreeding affected larval survival and larval development time but also unexpectedly affected maternal traits (clutch size and delay until laying), suggesting that females adjust clutch size and the delay until laying in response to being related to their mate.

## Introduction

Inbreeding is the mating between related individuals (Wright, 1977), which often leads to a reduction in fitness referred to as inbreeding depression (Charlesworth & Charlesworth, 1987; Charlesworth & Willis, 2009). Inbreeding depression is due to a general loss of heterozygosity, which may reduce offspring fitness either by increasing the risk that recessive deleterious alleles are expressed (partial dominance hypothesis; Charlesworth & Charlesworth, 1987, 1999) or by reducing the production of heterozygotes in situations where there is heterozygote advantage (overdominance hypothesis; Charlesworth & Charlesworth, 1987, 1999). Inbreeding depression has become widely recognized as a significant evolutionary force that may drive the evolution of mate choice (Blouin & Blouin, 1988) and mating systems (Charlesworth & Charlesworth, 1979; Lande & Schemske, 1985; Charlesworth *et al.*, 1990). Furthermore, inbreeding has important implications for agriculture because it can have detrimental effects on food production (Kristensen & Sørensen, 2005) and for conservation because it can increase the risk of extinction of local populations (Crnokrak & Roff, 1999; Keller & Waller, 2002). Therefore, understanding the mechanisms and consequences of inbreeding depression is a

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vital subject area in evolutionary biology (Charlesworth & Charlesworth, 1987; Charlesworth & Willis, 2009).

For inbreeding depression to occur, there must be directional dominance; that is, the deleterious alleles causing inbreeding depression must be biased towards one side of the trait mean (Wright, 1977; Lynch & Walsh, 1998). When there is directional dominance, the mean value of a trait will change as a consequence of inbreeding (Lynch & Walsh, 1998). Inbreeding depression can affect traits across the entire life cycle of an organism (Charlesworth & Charlesworth, 1987). Thus, the detrimental effects of inbreeding can extend to later in life, such as when inbred individuals reproduce as adults. When this is the case, the fitness of an individual can be influenced by both its own inbreeding coefficient (offspring inbreeding) and the inbreeding coefficient of its mother (maternal inbreeding). Indeed, in some species, maternal inbreeding may have as large an influence on offspring fitness as offspring inbreeding (Mattey *et al.*, 2013). Offspring and maternal inbreeding may have differential effects on traits depending on whether they are under offspring or maternal control. Traits that are expressed in the offspring, such as offspring growth and survival, can be regarded as offspring traits, whereas traits that are under maternal control, such as the number and size of eggs, can be regarded as maternal traits (Wilson *et al.*, 2005). Offspring inbreeding is likely to influence offspring traits only given that offspring cannot influence the number and size of eggs produced by their mothers. In contrast, maternal inbreeding may affect both maternal and offspring traits, given the potential for maternal effects on offspring growth and survival (Mousseau & Fox, 1998). For example, maternal inbreeding could affect offspring growth and survival through its effects on maternally controlled traits, such as egg size, laying and hatching patterns and maternal care (e.g. McParland *et al.*, 2007). Despite their potential importance, few studies have investigated differential effects of offspring and maternal inbreeding on offspring and maternal traits associated with early offspring fitness (Walling *et al.*, 2011; Mattey *et al.*, 2013; Huisman *et al.*, 2016). Neglecting effects of inbreeding on these traits could lead to an underestimation of the fitness costs of inbreeding if they cause early mortality of poor quality inbred individuals.

Prior work on the effects of maternal inbreeding has focused on the offspring's performance after laying or birth, and little is known about how maternal and offspring inbreeding affects egg size (Szulkin *et al.*, 2007; Fox, 2013) and patterns of egg laying or hatching. This is unfortunate given that these traits often have profound effects on the offspring's subsequent performance, thereby potentially explaining the detrimental effects of maternal inbreeding. For example, if inbred females lay smaller eggs, this can have detrimental fitness effects given that egg size often is positively associated with offspring growth and survival (birds: Krist, 2011; fish: Heath & Blouw, 1998; arthropods: Fox & Czesak, 2000). Similarly, patterns of egg laying or hatching can affect offspring survival, time to independence and size at independence (Stoleson & Beissinger, 1995; Stenning, 1996; Ford & Smiseth, 2016), thereby exacerbating the severity of inbreeding depression in offspring (de Boer *et al.*, 2015). Thus, if inbred females lay clutches that hatch more asynchronously, this can also have detrimental effects on offspring performance.

Here, we investigate the effect of maternal and offspring inbreeding on breeding success and maternal and offspring traits associated with early offspring performance in the burying beetle *Nicrophorus vespilloides* (Herbst). In this species, offspring suffer significant fitness costs across their life cycle when either the offspring themselves or their mothers are inbred (Mattey *et al.*, 2013; Pilakouta *et al.*, 2015, 2016; Pilakouta & Smiseth, 2016). *Nicrophorus vespilloides* is an excellent system to study the effects of inbreeding on egg size and egg laying because the eggs can be measured accurately and the timing of egg laying can be reliably recorded (Ford & Smiseth, 2016, 2017). This species breeds on small vertebrate carcasses, which are buried underground (Scott, 1998). Females lay eggs in the surrounding soil (Pukowski, 1933), and the eggs hatch asynchronously over a period of 16–56 h (Müller & Eggert, 1990; Smiseth *et al.*, 2006). The larvae self-feed directly off the carcass but also beg for predigested carrion from the parents (Eggert *et al.*, 1998). The larvae reach independence around 6 days after hatching and disperse into the soil where they pupate and ultimately eclose as adults (Smiseth *et al.*, 2006). Here, we manipulate the level of inbreeding of females producing outbred offspring (maternal inbreeding) and the level of inbreeding of offspring produced by outbred mothers (offspring inbreeding). This design allows us to test for differential effects of maternal and offspring inbreeding on maternal and offspring traits associated with early offspring performance.

It has generally been assumed that inbreeding depression is a linear function of the inbreeding coefficient (Lynch & Walsh, 1998). However, inbreeding may have nonlinear effects that would only be detected if three or more groups of individuals with different inbreeding coefficients are examined. Few studies have experimentally tested for a nonlinear effect of inbreeding, and the majority of those that have done so have focused on domestic cattle (Hudson & Van Vleck, 1984; Miglior *et al.*, 1992; Thompson *et al.*, 2000; Biffani *et al.*, 2002; Sørensen *et al.*, 2006; Croquet *et al.*, 2007; Gulisija *et al.*, 2007). Here, we use three levels of inbreeding to test for a nonlinear relationship between the inbreeding coefficient and the magnitude of inbreeding depression: outbred (the focal individual's parents were unrelated at the grandparent level; $F \approx 0.00$), moderately inbred (the focal individual's parents were

cousins; F ≈ 0.125) and highly inbred (the focal individual's parents were siblings; F ≈ 0.25). We measured effects of maternal and offspring inbreeding on breeding success (number of dispersing larvae), maternal traits (egg laying patterns and egg size) and offspring traits (i.e. larval survival and mass at independence). We expected both maternal and offspring inbreeding to lead to a reduction in breeding success. We expected maternal inbreeding to influence both maternal and offspring traits, given the potential for maternal effects on offspring growth and survival (Mousseau & Fox, 1998). Conversely, we expected offspring inbreeding to influence offspring traits only given that offspring cannot influence the number and size of eggs produced by their mothers.

## Materials and methods

### Study animals

The beetles used in this study were third- to sixth-generation beetles from an outbred laboratory population maintained at the University of Edinburgh. The stock population descended from wild beetles caught at Corstorphine Hill, Edinburgh, UK (55.9500° N, 3.2833° W). To ensure that we had full control over the pedigree of our stock population, we housed all beetles in individual transparent plastic boxes (124 mm × 82 mm × 22 mm) from the day that they eclosed as adults. Furthermore, when beetles were paired for breeding, we mated each female with a single male of known identity to prevent paternity uncertainty. We therefore knew the identity of the ancestors of every beetle in our laboratory population dating back to the wild-caught beetles (Mattey & Smiseth, 2015). Keeping beetles in individual boxes from dispersal ensured that all experimental beetles were virgins at the start of the experiments. We ensured that the laboratory population was outbred by maintaining a large population each generation (86–98 broods per generation), recruiting three offspring only from each family to the next generation, outcrossing the laboratory population with wild-caught beetles each summer, and never mating closely related males and females (i.e. siblings or cousins; Mattey & Smiseth, 2015; Mattey *et al.*, in press). These practices ensured that the inbreeding coefficient, F, in our stock population was very low (mean for individuals in the sixth generation: $F = 0.0002$; Mattey *et al.*, in press). Beetles were kept at 20 ± 2°C (mean ± range) under a 16:8 light:dark cycle and were fed small pieces of organic beef twice a week. All beetles were sexually mature, virgins and of prime reproductive age (10–28 days post-eclosion) at the start of the experiments.

### Experimental procedures

For the experiment on the effects of maternal inbreeding, we generated experimental females with the following three levels of inbreeding: (1) outbred females, produced by mating a given female's mother with an unrelated male (i.e. a male with whom the mother did not share a common grandparent or a closer relative; $F \approx 0.00$); (2) moderately inbred females, produced by mating a given female's mother with a cousin (i.e. a male with whom the mother shared two common grandparents; $F \approx 0.125$); and (3) highly inbred females, produced by mating a given female's mother with a brother (i.e. a male with whom the mother shared both parents; $F \approx 0.25$). We always mated experimental females to an unrelated outbred male (i.e. a male with whom the female had no shared grandparents). We did this to ensure that the offspring in this experiment always were outbred, such that any subsequent effects of the experimental treatments could be attributed to the inbreeding status of the experimental females.

For the experiment on the effects of offspring inbreeding, we generated experimental broods of offspring with the following three levels of inbreeding: (1) outbred broods, produced by mating the brood's mother with an unrelated male (i.e. a male with whom the mother did not share a common grandparent or a closer relative; $F \approx 0.00$); (2) moderately inbred broods, produced by mating the brood's mother with a cousin (i.e. a male with whom the mother shared two common grandparents; $F \approx 0.125$); and (3) highly inbred broods, produced by mating the brood's mother with a brother (i.e. a male with whom the mother shared both parents; $F \approx 0.25$). In this experiment, we used outbred females only to ensure that any subsequent effects could be attributed to the inbreeding status of the offspring.

Different individuals were used in the maternal inbreeding and offspring inbreeding experiments. We carried out both experiments in batches of up to 20 broods at a time with the three levels of inbreeding represented in all batches to minimize any confounding effects due to potential differences between batches. At the start of the experiments, we placed the breeding female together with a male in a Petri dish (90 mm × 12 mm) for 4 h to allow them time to mate (Botterill-James *et al.*, 2017). We then transferred each female to a clear breeding box (170 mm × 120 mm × 60 mm) containing 1 cm of moist compost and a previously frozen mouse carcass weighing 23.06 g ± 2.26 (mean ± SD, supplied from Livefoods Direct Ltd, Sheffield, UK). The male was removed at this stage because the presence or absence of the male does not affect offspring fitness under laboratory conditions (Smiseth *et al.*, 2005). In *N. vespilloides*, eggs are visible at the bottom of the breeding box and can be seen on images obtained by placing the boxes on flatbed scanners (Canon CanoScan 9000F Mark II; Canon Inc., Tokyo, Japan; Ford & Smiseth, 2016). In the small amount of soil used, the number of eggs that are visible is strongly correlated with the actual clutch size (Monteith

*et al.*, 2012). We scanned the breeding boxes every hour using VueScan professional edition software (Hamrick Software, Sunny Isles Beach, FL, USA). From the scanned images, we counted the number of new eggs laid each hour to determine laying spread and laying skew (see below) and clutch size (the number of eggs laid).

In *N. vespilloides*, females determine the hatching pattern through their timing of laying (Smiseth *et al.*, 2006), and we can therefore use laying time as a proxy for hatching time as the former can be determined accurately from the scans. The laying pattern can be described in two ways: the time between the first and last egg being laid, which is termed 'laying spread' (Smiseth *et al.*, 2006; Takata *et al.*, 2015); and the extent to which laying is skewed towards the earlier part of the laying period, which is termed 'laying skew' (Smiseth *et al.*, 2008). We calculated a laying skew index using the formula $\Sigma((t_i - t_m)/t_m)p_i$, where $p_i$ is the proportion of the total clutch that is laid in a given scan, $t_i$ is the time interval of a given scan in relation to the initiation of oviposition and $t_m$ is the middle of the laying period (Smiseth *et al.*, 2008; Ford & Smiseth, 2016). Prior work shows that this index is usually negative, indicating that egg laying is normally skewed towards the first half of the laying period. Values closer to −1 represent a more asymmetric laying skew where a larger proportion of eggs are laid early on, whereas values closer to 0 represent a more symmetrical laying skew. Additionally, we measured egg size of the first five eggs that were lying flat against the bottom of the box in each clutch using ImageJ (Abramoff *et al.*, 2004). Eggs were measured from the second image on which they were present given that they appear to shrink somewhat during the first hour after being laid (personal observation). For each egg, we measured its length and width three times, and the mean length and width were used to calculate a prolate spheroid volume (V) using the equation $V = (1/6)\pi w^2 L$, where w is the width and L the length of the egg (Berrigan, 1991). We continued to check the scans after hatching started to gauge the number of unhatched eggs.

To monitor offspring performance, we checked the boxes for dispersal each day. At the time of dispersal, we counted the number of larvae in the brood and weighed the entire brood. After dispersal, we measured the pronotum width of each female with a Mitutoyo Digimatic Calliper. We confirm that there was no difference in the pronotum width of females depending on their inbreeding status (ANOVA: $F_2 = 1.00$, $P = 0.381$). We initially set up 178 pairs across our two experiments. However, 20 were excluded due to technical problems with the scanners; 23 were excluded because females did not lay any eggs; 37 were excluded from analyses of larval mass and development time because, although females laid eggs, no larvae reached dispersal; and one was excluded because the female started laying too late to allow measurement of laying spread, laying skew or clutch size. The final sample sizes (i.e. number of pairs) in each treatment were as follows: outbred offspring $n = 21$, moderately inbred offspring $n = 24$, highly inbred offspring $n = 26$, outbred mother $n = 26$, moderately inbred mother $n = 26$ and highly inbred mother $n = 28$.

### Statistical analyses

We carried out the statistical analyses in R 3.3.1 (R Core Team, 2014). We first tested for effects of maternal and offspring inbreeding on breeding success; that is, the number of dispersing larvae, defined as the number of larvae in the brood at the time of dispersal. We then carried out analyses using the following nine traits (five maternal traits and four offspring traits): (1) Clutch size, defined as the total number of eggs laid before the first egg hatched (Müller *et al.*, 1990). (2) Delay until the onset of laying, defined as the number of hours after the female was placed on the carcass until the first egg was laid. (3) Laying skew index as defined above. (4) Laying spread, defined as the number of hours between the first and last egg being laid. (5) Egg size, calculated as the average volume in $mm^3$ of five eggs in each clutch. (6) Hatching success, based on counts of the number of eggs hatching and the number of unhatched eggs. We did the analyses in two ways; that is, when including all clutches and when excluding clutches where no eggs hatched. (7) Larval survival, based on the number of larvae surviving until dispersal and the number of larvae dying between hatching and dispersal. We did the analyses in two ways; that is, when including all broods where eggs hatched and when excluding broods where eggs hatched but no larvae survived to dispersal. To ensure that all of the data were retained and the response was as informative as possible, we used the *cbind* function to bind the number of successes and failures for both hatching success and larval survival. That is, for hatching success, we used the number of eggs hatching vs. the number of unhatched eggs, whereas for larval survival, we used the number of larvae dispersing from the carcass vs. the number of larvae that died between hatching and dispersal. (8) Larval development time, calculated as the time in hours from when the first egg in the clutch was predicted to hatch until the larvae dispersed into the soil. We calculated the predicted time of hatching for the first egg by adding the average duration of egg development, which is 59 h (Smiseth *et al.*, 2006), to the information on the onset of laying (see above). (9) Average larval mass, calculated as the mass of the brood at the time of dispersal divided by the number of larvae in the brood. We analysed data on the effects of maternal inbreeding and offspring inbreeding separately, using the same procedures for both. We carried out a separate generalized linear model (GLM) to test for an effect of maternal and offspring inbreeding status on each of the traits above. The model family used for each model is

listed in Tables 1 and 2. For every GLM, we conducted a Fligner–Killeen test to check for homogeneity of variance. We also checked that the residuals were normally distributed and that there were no unduly influential observations (i.e. no Cook's distances >1). Because we carried out multiple tests, we applied a Benjamini–Hochberg procedure to the model results from each experiment. This did not lead to a change in the interpretation of any of the results.

## Results

### Maternal inbreeding

Maternal inbreeding had a significant negative effect on breeding success, reflecting that highly inbred females produced broods with a significantly smaller number of dispersing larvae than outbred females (Table 1). As expected, there were significant effects of maternal inbreeding on both maternal and offspring traits. Firstly, maternal inbreeding had a significant effect on laying skew (Table 1). The majority of clutches had a negative laying skew index (92% for clutches laid by outbred females, 86% for moderately inbred females and 81% for highly inbred females), indicating that most of the eggs in a given clutch were laid during the first half of the laying period. This index was closer to 0 for clutches laid by highly inbred females than for clutches produced by outbred females (Table 1, Fig. 1a). Thus, highly inbred females produced clutches where the eggs were laid more symmetrically around the middle of the laying period than did outbred females. There was no evidence that maternal inbreeding had an effect on any of the other maternal traits (Table 1). Secondly, maternal inbreeding had a significant effect on hatching success when we excluded broods where all eggs failed to hatch, although

**Table 1** Results of GLMs testing for effects of maternal inbreeding on breeding success (number of dispersing larvae), maternal traits (clutch size, delay until laying, laying skew, laying spread and egg size) and offspring traits (hatching success, larval survival, larval development time and larval mass). Significant effects are shown in bold.

| | | | | | Overall effect of maternal inbreeding | | Moderately inbred compared to outbred | | Highly inbred compared to outbred | |
|---|---|---|---|---|---|---|---|---|---|---|
| Trait | Model error distribution | Mean outbred (±SD) | Mean moderately inbred (±SD) | Mean highly inbred (±SD) | Test statistic | *P* | Test statistic | *P* | Test statistic | *P* |
| Number of dispersing larvae | Negative binomial | 15.47 (8.83) | 13.41 (6.73) | 8.74 (6.23) | $X^2_{2,55} = 7.52$ | **0.023** | $z = -0.70$ | 0.482 | $z = -2.65$ | **0.008** |
| Clutch size | Negative binomial | 30.40 (9.45) | 31.76 (8.21) | 30.81 (8.78) | $X^2_{2,64} = 0.27$ | 0.872 | $z = 0.52$ | 0.606 | $z = 0.16$ | 0.875 |
| Delay until laying (h) | Inverse Gaussian | 18.72 (7.94) | 15.76 (7.14) | 17.76 (7.55) | $F_{2,64} = 0.74$ | 0.479 | $t = 1.32$ | 0.193 | $t = 0.40$ | 0.693 |
| Laying skew | Gaussian | −0.35 (0.22) | −0.24 (0.26) | −0.14 (0.19) | $F_{2,64} = 4.88$ | **0.011** | $t = 1.62$ | 0.110 | $t = 3.12$ | **0.003** |
| Laying spread (h) | Gaussian | 51.40 (18.53) | 51.67 (15.33) | 41.43 (17.38) | $F_{2,64} = 2.48$ | 0.092 | $t = 0.05$ | 0.958 | $t = -1.96$ | 0.055 |
| Egg size ($mm^3$) | Inverse Gaussian | 1.72 (0.20) | 1.82 (0.19) | 1.77 (0.28) | $F_{2,60} = 0.91$ | 0.409 | $t = -1.34$ | 0.184 | $t = -0.65$ | 0.520 |
| Hatching success including failures | Quasibinomial | 0.77 (0.40) | 0.92 (0.11) | 0.84 (0.14) | $X^2_{2,64} = 4.38$ | 0.112 | $t = 1.96$ | 0.054 | $t = 0.22$ | 0.825 |
| Hatching success excluding failures | Quasibinomial | 0.96 (0.07) | 0.92 (0.11) | 0.84 (0.14) | $X^2_{2,59} = 15.73$ | **0.0004** | $t = -1.06$ | 0.295 | $t = -3.64$ | **0.0006** |
| Larval survival including failures | Quasibinomial | 0.38 (0.31) | 0.36 (0.27) | 0.18 (0.21) | $X^2_{2,59} = 8.14$ | **0.017** | $t = -0.19$ | 0.851 | $t = -2.49$ | **0.016** |
| Larval survival excluding failures | Quasibinomial | 0.47 (0.27) | 0.45 (0.22) | 0.32 (0.18) | $X^2_{2,42} = 3.12$ | 0.210 | $t = -0.44$ | 0.664 | $t = -1.68$ | 0.100 |
| Larval development time (h) | Inverse Gaussian | 140.69 (17.82) | 142.12 (15.34) | 150.83 (11.61) | $F_{2,42} = 1.60$ | 0.213 | $t = -0.27$ | 0.789 | $t = -1.68$ | 0.101 |
| Larval mass (g) | Gaussian | 0.22 (0.06) | 0.21 (0.05) | 0.23 (0.12) | $F_{2,55} = 0.35$ | 0.707 | $t = -0.66$ | 0.513 | $t = 0.07$ | 0.944 |

 

**Table 2** Results of GLMs testing for effects of offspring inbreeding on breeding success (number of dispersing larvae), maternal traits (clutch size, delay until laying, laying skew, laying spread and egg size) and offspring traits (hatching success, larval survival, larval development time and larval mass). Significant effects are shown in bold.

| | | | | | Overall effect of offspring inbreeding | | Moderately inbred compared to outbred | | Highly inbred compared to outbred | |
|---|---|---|---|---|---|---|---|---|---|---|
| Trait | Model error distribution | Mean outbred (±SD) | Mean moderately inbred (±SD) | Mean highly inbred (±SD) | Test statistic | *P* | Test statistic | *P* | Test statistic | *P* |
| Number of dispersing larvae | Negative binomial | 7.94 (6.18) | 12.47 (8.15) | 8.89 (5.67) | $X^2_{2,51}$ = 4.33 | 0.115 | *z* = 1.97 | **0.049** | *z* = 0.50 | 0.618 |
| Clutch size | Negative binomial | 37.94 (15.16) | 31.04 (9.48) | 31.08 (7.83) | $X^2_{2,64}$ = 6.10 | **0.047** | *z* = −2.17 | **0.030** | *z* = −2.17 | **0.030** |
| Delay until laying (h) | Inverse Gaussian | 19.44 (8.37) | 26.63 (23.65) | 32.92 (23.99) | $F_{2,64}$ = 3.04 | 0.055 | *t* = −1.78 | 0.080 | *t* = −2.23 | **0.029** |
| Laying skew | Gaussian | −0.12 (0.26) | −0.17 (0.21) | −0.18 (0.28) | $F_{2,63}$ = 0.36 | 0.696 | *t* = −0.67 | 0.504 | *t* = −0.82 | 0.418 |
| Laying spread (h) | Inverse Gaussian | 45.17 (27.39) | 39.42 (16.56) | 35.92 (16.94) | $F_{2,64}$ = 1.08 | 0.347 | *t* = 0.87 | 0.389 | *t* = 1.51 | 0.137 |
| Egg size ($mm^3$) | Inverse Gaussian | 1.94 (0.30) | 1.97 (0.27) | 2.03 (0.34) | $F_{2,61}$ = 0.42 | 0.660 | *t* = −0.27 | 0.789 | *t* = −0.85 | 0.398 |
| Hatching success including failures | Quasibinomial | 0.85 (0.25) | 0.89 (0.17) | 0.83 (0.20) | $X^2_{2,64}$ = 0.61 | 0.737 | *t* = 0.66 | 0.515 | *t* = −0.03 | 0.980 |
| Hatching success excluding failures | Quasibinomial | 0.90 (0.14) | 0.89 (0.17) | 0.83 (0.20) | $X^2_{2,63}$ = 1.64 | 0.441 | *t* = −0.45 | 0.657 | *t* = −1.24 | 0.220 |
| Larval survival including failures | Quasibinomial | 0.25 (0.24) | 0.33 (0.30) | 0.24 (0.27) | $X^2_{2,63}$ = 2.26 | 0.323 | *t* = 1.46 | 0.148 | *t* = 0.67 | 0.508 |
| Larval survival excluding failures | Quasibinomial | 0.28 (0.24) | 0.46 (0.25) | 0.36 (0.26) | $X^2_{2,47}$ = 6.00 | **0.049** | *t* = 2.34 | **0.024** | *t* = 0.82 | 0.415 |
| Larval development time (h) | Gaussian | 158.00 (37.86) | 132.47 (28.68) | 137.28 (17.60) | $F_{2,47}$ = 3.53 | **0.037** | *t* = −2.52 | **0.015** | *t* = −2.07 | **0.044** |
| Larval mass (g) | Gaussian | 0.23 (0.03) | 0.21 (0.03) | 0.20 (0.04) | $F_{2,50}$ = 1.86 | 0.166 | *t* = −1.06 | 0.292 | *t* = −1.93 | 0.060 |

not when we included these broods (Table 1). When we excluded clutches with total hatching failure, highly inbred females produced eggs with a significantly lower hatching success than outbred females (Table 1, Fig. 1b). Maternal inbreeding significantly affected larval survival when we included broods where no larvae dispersed but not when we excluded these broods (Table 1). When we included broods where no larvae dispersed, fewer larvae survived in broods produced by highly inbred mothers compared to broods produced by outbred mothers. The lack of an effect of maternal inbreeding on larval survival when we excluded broods where no larvae dispersed suggests that this effect was driven by a greater number of total brood failures when the mother was highly inbred (no larvae reached dispersal in nine broods when the mother was highly inbred compared to four broods when the mother was moderately inbred and four when she was outbred). There were no effects of maternal inbreeding on any other offspring traits (Table 1). Thus, our results suggest that highly inbred females had a lower breeding success because their eggs had a lower hatching success and their larvae suffered greater mortality, rather than because they produced a smaller number of eggs.

## Offspring inbreeding

Although there was no overall effect of offspring inbreeding on breeding success, there were a greater number of dispersing larvae when offspring were moderately inbred than when they were outbred (Table 2). Contrary to what we expected, there were significant effects of offspring inbreeding on both maternal and offspring traits. Firstly, offspring inbreeding had a significant effect on both clutch size and onset of egg laying (Table 2). Females that were mated to an unrelated male (i.e. females producing outbred eggs) laid a greater number of eggs than females that were mated to their brother or cousin (i.e. females producing highly inbred and moderately inbred eggs, respectively) (Table 2). Likewise, females that were mated to their brother or cousin took on average longer to begin laying eggs than females that were mated to an unrelated male (Table 2). Given that offspring cannot influence the number and size of eggs produced by their mothers, these findings suggest that females adjust decisions about how many eggs to lay and when to start laying based on whether they mated with a related or an unrelated male. There was no effect of offspring inbreeding on any of the other

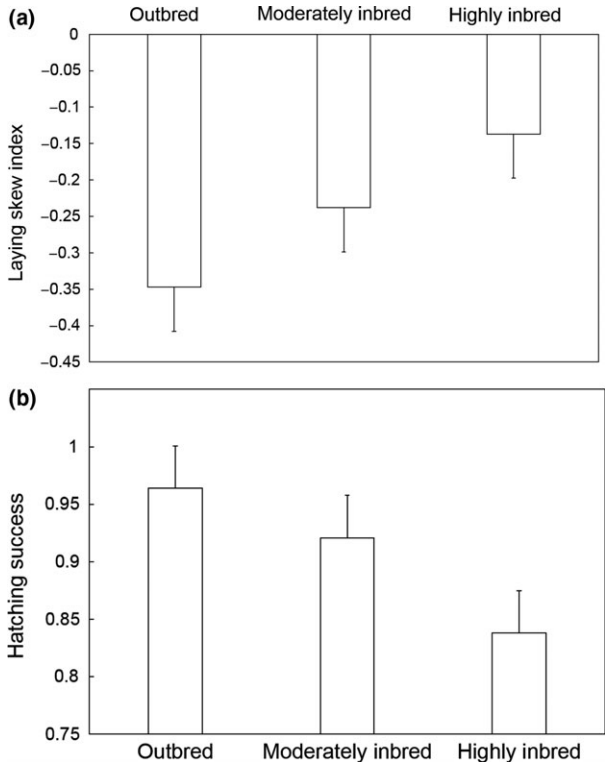


**Fig. 1** The effect of maternal inbreeding on (a) laying skew index (an index calculated based on the extent to which laying is skewed towards the earlier part of the laying period) and (b) hatching success (the proportion of the clutch that hatched, excluding clutches where eggs were laid but none hatched). Error bars represent standard errors.

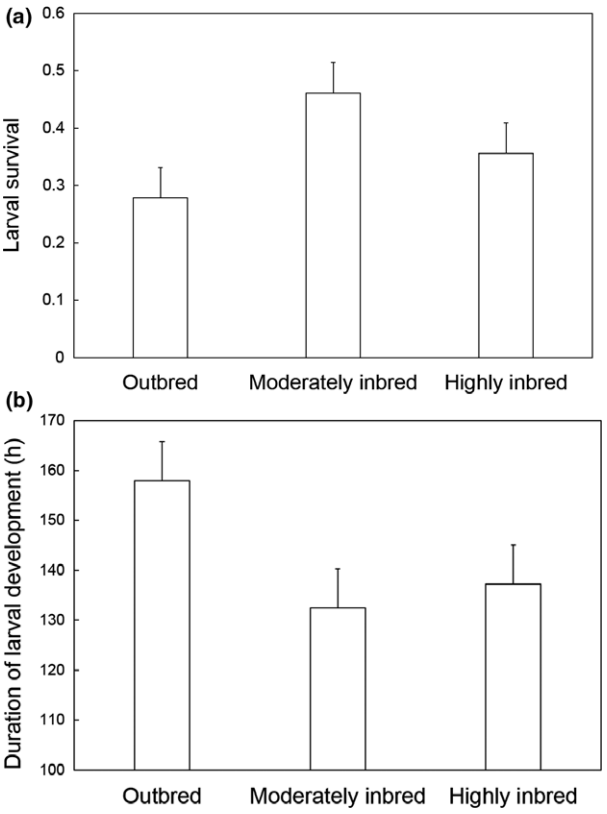


**Fig. 2** The effect of offspring inbreeding status on (a) larval survival from hatching to dispersal (the proportion of the larvae hatching that survived to dispersal, excluding broods where larvae hatched but none dispersed) and (b) the duration of larval development (the number of hours from the estimated timing of the start of hatching until dispersal). Error bars represent standard errors.

maternal traits (Table 2). Secondly, offspring inbreeding had a significant effect on two offspring traits: larval survival from hatching to dispersal and larval development time (Table 2). Moderately inbred larvae had higher survival than outbred larvae when we excluded broods where no larvae dispersed (Table 2, Fig. 2a). Furthermore, moderately and highly inbred larvae developed significantly faster than outbred larvae (Table 2, Fig. 2b). There was no effect of offspring inbreeding on hatching success (Table 2). Thus, keeping in mind that females mated to an unrelated male (i.e. females producing outbred eggs) laid a greater number of eggs than females mated to a related male, our results suggest that the greater breeding success when offspring were moderately inbred was associated with high larval survival from hatching to dispersal (Table 2).

## Discussion

We investigated the effects of both maternal and offspring inbreeding using an experimental design with three levels of maternal and offspring inbreeding (i.e. $F \approx 0.00$, $F \approx 0.125$ and $F \approx 0.25$). Firstly, we find evidence that both maternal inbreeding and offspring inbreeding affected breeding success. As expected, highly inbred females produced broods with significantly fewer dispersing larvae than outbred females. In contrast, there was no overall effect of offspring inbreeding on breeding success, although there were a greater number of dispersing larvae when offspring were moderately inbred than when they were outbred. Secondly, we find evidence for differential effects of maternal inbreeding and offspring inbreeding on maternal and offspring traits. Maternal inbreeding affected laying skew, hatching success and larval survival, whereas offspring inbreeding affected clutch size, delay until onset of egg laying, larval survival and larval development time. Our results confirm that maternal inbreeding affected both maternal and offspring traits, which is in line with our expectations given that female parents can influence offspring traits through maternal effects. However, our results suggest that offspring inbreeding also affected both maternal and offspring

traits, which is surprising given that offspring cannot influence the number of eggs produced by their mothers or the timing of egg laying. Thus, this finding suggests that females alter their decisions about the number of eggs they lay and the timing of egg laying based on whether they are mated with a related or an unrelated male. Finally, we found evidence of a nonlinear effect of the level of offspring inbreeding on larval survival, with moderately inbred offspring experiencing the greatest survival from hatching to independence. Below, we discuss the wider implications of these results for our understanding of the effects of maternal and offspring inbreeding on breeding success and offspring performance.

We found that maternal inbreeding had a negative effect on breeding success with highly inbred females producing fewer dispersing larvae than outbred females. This finding is largely consistent with prior work reporting negative effects of maternal inbreeding on breeding success and early offspring performance in *N. vespilloides* (Mattey *et al.*, 2013) as well as in birds (Keller, 1998; Jamieson *et al.*, 2003; Reid *et al.*, 2003; Richardson *et al.*, 2004; Szulkin *et al.*, 2007) and mammals (Huisman *et al.*, 2016). We recorded effects of maternal inbreeding on maternal and offspring traits associated with early offspring performance, which allowed us to identify at least some of the potential mechanisms by which maternal inbreeding causes a reduction in breeding success. We found that maternal inbreeding affected laying skew, hatching success and larval survival. As discussed below, highly inbred females produced clutches that had a laying skew index that was closer to 0 (i.e. egg laying was less skewed towards the beginning of the laying period) than did outbred females. We also found that a laying skew index closer to 0 was associated with higher offspring survival, suggesting that the effect of maternal inbreeding on hatching skew cannot account for lower breeding success of highly inbred females. We found that highly inbred females laid eggs that had a significantly lower hatching success compared to eggs laid by outbred females when we excluded clutches where no eggs hatched. Furthermore, fewer larvae survived from hatching to dispersal in broods produced by highly inbred females compared to those produced by outbred females. In contrast, there was no evidence that maternal inbreeding affected clutch size, delay until laying, laying spread, egg size, larval growth or larval development time. Thus, our results suggest that the detrimental effects of maternal inbreeding on breeding success are mediated through a reduction in hatching success of eggs laid by highly inbred females and greater mortality of larvae produced by highly inbred mothers rather than a reduction in clutch size or egg size.

As mentioned above, we found that highly inbred females produced clutches that had a hatching skew index that was closer to 0 than outbred females. In other words, highly inbred females produced clutches where egg laying was less skewed towards the beginning of the laying period than did outbred females. This finding is contrary to what we anticipated if the detrimental effects of maternal inbreeding on breeding success were mediated through an effect on hatching skew. The reason for this is that a hatching skew index closer to 0 is assumed to be associated with increased rather than reduced offspring survival. We conducted a post hoc test of this assumption using data from the experiment on the effects of offspring inbreeding (we used these data because all mothers were outbred). We found that a more negative value of the laying skew index (i.e. when egg laying was more strongly skewed towards the beginning of the laying period) was associated with reduced larval survival (Pearson's correlation, $t_{46} = 3.07$, $P = 0.004$, $r = 0.41$) as well as with fewer dispersing larvae ($t_{47} = 2.74$, $P = 0.009$, $r = 0.37$). This effect is presumably mediated through an effect of laying skew on sibling competition. There is good evidence from studies on birds that asynchronous hatching is associated with asymmetric sibling competition (Magrath, 1990; Stoleson & Beissinger, 1995; Mock & Parker, 1997), where early-hatched offspring are competitively superior to offspring that hatch later (Lack, 1947; Stinson, 1979). Likewise, studies on *N. vespilloides* and the closely related *Nicrophorus quadripunctatus* find that late-hatched larvae have reduced growth rates and are less likely to survive to independence than early-hatched larvae (Smiseth *et al.*, 2007; Takata *et al.*, 2013). Thus, the finding that highly inbred females produce clutches where egg laying was less skewed towards the beginning of the laying period suggests that these females adjust their laying patterns facultatively and that by doing so, they partially mitigate some of the negative effects of maternal inbreeding on breeding success. However, we note that females cannot completely compensate for the detrimental effect of maternal inbreeding given that the number of larvae reaching dispersal is still lower for broods produced by highly inbred females.

Although there was no overall effect of offspring inbreeding on breeding success, we found that a greater number of larvae reached dispersal in broods where offspring were moderately inbred compared to broods where they were outbred. We also found that females that were mated to related males (and thus were producing inbred offspring) laid fewer eggs than those that were mated to unrelated males (and thus producing outbred offspring). This finding is consistent with a recent theoretical model predicting that parents producing inbred offspring should produce fewer offspring and invest more resources in individual offspring (Duthie *et al.*, 2016). Additionally, we found that females producing highly inbred offspring took longer to begin egg laying after encountering the carcass. It is unlikely that this would be beneficial for the offspring given that this

delay would be associated with an increase in microbial growth over time, which reduces egg survival (Jacobs *et al.*, 2014). We found that inbred larvae developed more quickly from hatching to dispersal than outbred larvae but attained a similar average mass at dispersal. Highly inbred larvae dispersed around 21 h sooner after hatching than outbred larvae, which more than counteracted the 13-h delay in the onset of laying by females producing highly inbred offspring. Our study provides no information on the mechanism behind the shorter development time for highly inbred larvae. However, a recent study on the same species found that outbred females provide more direct care towards inbred larvae, resulting in inbred and outbred larvae attaining the same mass at dispersal (Mattey *et al.* in press). Thus, it is possible that an increase in direct care towards inbred larvae allows them to develop faster, which would be beneficial for the offspring because the carcass becomes increasingly inhospitable for larvae as decomposition occurs (Rozen *et al.*, 2008). If so, this could contribute to the greater larval survival that we observed in moderately inbred broods, leading to a greater number of larvae at dispersal.

Finally, our results provide evidence of a nonlinear effect of offspring inbreeding on breeding success. We found that a greater number of larvae reached dispersal in broods of moderately inbred larvae compared to broods of either outbred or highly inbred larvae. Likewise, moderately inbred larvae had greater survival than either outbred or highly inbred larvae when excluding broods where no larvae dispersed. Previous studies on this species have found evidence for an effect of offspring inbreeding on offspring survival (Mattey *et al.*, 2013; Pilakouta *et al.*, 2015, 2016; Pilakouta & Smiseth, 2016). We note that these studies also found detrimental effects of offspring inbreeding on other components of offspring performance, such as survival from dispersal to eclosion (Mattey *et al.*, 2013; Pilakouta *et al.*, 2015, 2016; Pilakouta & Smiseth, 2016), survival from hatching to eclosion (Pilakouta *et al.*, 2016) and adult lifespan (Pilakouta *et al.*, 2015; Pilakouta & Smiseth, 2016). Our design differs from that used in the majority of laboratory studies investigating inbreeding depression, which simply compare fitness-related traits of outbred individuals with experimentally generated inbred individuals (with the inbreeding coefficient of the inbred treatment varying between studies) (Lynch & Walsh, 1998). Meanwhile, studies on inbreeding depression in the field often determine inbreeding coefficients from a pedigree, often assuming that inbreeding depression is a linear function of the inbreeding coefficient (Lynch & Walsh, 1998). Nevertheless, there is some evidence for nonlinear effects of inbreeding from studies on domestic cattle (Hudson & Van Vleck, 1984; Miglior *et al.*, 1992; Thompson *et al.*, 2000; Biffani *et al.*, 2002; Sørensen *et al.*, 2006; Croquet *et al.*, 2007; Gulisija *et al.*, 2007), and studies on mice find that offspring produced by intermediately related parents are larger than those produced by unrelated or closely related parents (Barnard & Fitzsimons, 1989; Keane, 1990). There is also some evidence from studies on humans that couples that are moderately related to each other have a greater number of children (Helgason *et al.*, 2008; Labouriau & Amorim, 2008). We suggest that such nonlinear effects of offspring inbreeding could arise as a consequence of maternal effects on offspring. There is evidence that maternal care buffers against the detrimental effect of offspring inbreeding in *N. vespilloides* (Pilakouta *et al.*, 2015). Furthermore, the smaller clutch sizes laid by females producing inbred offspring may lead to a lower initial number of larvae hatching, which would allow females to provide more care to each larva in inbred broods as predicted by a recent theoretical model (Duthie *et al.*, 2016). Thus, nonlinear effects of inbreeding could arise if females overcompensate for the detrimental effects of moderate offspring inbreeding by providing more care, resulting in higher larval survival for moderately inbred offspring compared to outbred offspring. Meanwhile, an increase in maternal care may just be sufficient to mitigate the detrimental effects of inbreeding when offspring are highly inbred, resulting in similar survival of highly inbred and outbred offspring (Mattey *et al.*, in press).

In conclusion, we found that both maternal inbreeding and offspring inbreeding affected breeding success, that maternal inbreeding and offspring inbreeding affected different traits associated with early offspring performance and that there were nonlinear effects of offspring inbreeding. Our results provide novel insights into inbreeding by suggesting that maternal inbreeding and offspring inbreeding have differential effects on maternal and offspring traits. Maternal inbreeding affected laying skew, hatching success and larval survival, whereas offspring inbreeding affected clutch size, delay until onset of egg laying, larval survival and larval development time. Furthermore, our results suggest that inbred females facultatively adjust their laying patterns to compensate for some of the detrimental effects of maternal inbreeding on offspring. In support of this, we found that inbred females lay clutches with hatching skew index that was closer to 0 (i.e. the eggs were laid more symmetrically around the middle of the laying period), which is associated with greater offspring survival. Finally, we found evidence of a nonlinear effect of offspring inbreeding coefficient on the number of larvae dispersing, with the greatest number of larvae dispersing in moderately inbred broods, reflecting that these broods experienced the greatest larval survival. This result highlights the importance of considering deviations from linearity when testing for an effect of inbreeding, and we recommend that future studies incorporate multiple inbreeding treatments where possible to increase our

understanding of the effects of inbreeding and to gain an insight into the potential mechanisms behind these effects.

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Original Article

# Resource availability, but not polyandry, influences sibling conflict in a burying beetle *Nicrophorus vespilloides*

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Conflict over resources is a fundamental component of family life. Family conflicts are predicted to be strongly influenced by resource availability and levels of relatedness between family members. Here, we examined the effects of these factors on intra-brood sibling conflict in a family living beetle, *Nicrophorus vespilloides,* where offspring are partially dependent on parental provisioning. Specifically, we measured the intensity of offspring begging behavior in response to experimental manipulation of 1) relatedness between siblings (through mating females monogamously or polyandrously) and 2) resource availability (through varying the amount of resources at the onset of breeding). We found no effect of polyandry on sibling conflict or patterns of female reproductive investment, but we did find that sibling conflict was influenced by resource availability. Specifically, larvae spent more time begging on smaller carcasses, but only in smaller clutches. In addition, we found that resource availability affected patterns of female reproductive investment: when resource availability was low, females laid eggs more synchronously and produced fewer eggs but of a larger size. We discuss potential explanations for these results, and the implications of this study for understanding the factors that mediate family dynamics.

*Key words*: **burying beetle, laying asynchrony, *Nicrophorus vespilloides,* polyandry, resource availability, sibling conflict.**

## INTRODUCTION

Conflict over limited resources is a fundamental component of family life across a wide range of organisms (Mock and Parker 1997). Understanding how different biotic and abiotic factors mediate the resolution of conflict between family members and the consequences of this for the maintenance and diversification of animal societies is thus a key challenge for evolutionary biologists (Maynard Smith and Szathmary 1995; Bourke 2014). The majority of studies have addressed this challenge from the perspective of the factors that reduce inter-generational conflict; that is, factors that either reduce the costs of prolonged parental care to the parents or increase the benefits of care to their offspring (e.g. Emlen and Wrege 1992; Brown et al. 2010; Griffin et al. 2013). However, family life is also characterized by prolonged association between siblings (e.g. intra-generational associations) and the level of conflict and/or cooperation between siblings may be just as important as that between parents and offspring for the maintenance of family living (Falk et al. 2014; Ruch et al. 2014; Kramer and Meunier 2016). Despite this, the mechanisms underpinning intra-brood social interactions and their subsequent implications for the evolution of sociality are yet to be fully investigated (Forbes 2007; Falk et al. 2014; Ruch et al. 2014).

Like other forms of family interactions, the balance of conflict and cooperation between siblings should depend on the level of relatedness between brood mates and the relative costs and benefits of cooperative behavior (Hamilton 1964). Therefore, any factors that influence these traits should be important in mediating the maintenance and diversification of family living. Two factors are likely to play a key role in this context. First, low levels of polyandry (female mating with multiple males) should increase relatedness between siblings, thereby promoting increased cooperation (Briskie et al. 1994; Hughes et al. 2008; Cornwallis et al. 2010; Lukas and Clutton-Brock 2012). Second, for a given level of relatedness, resource availability should be important in mediating the costs and benefits of cooperating with group members. Specifically, as the intensity of sibling competition is largely driven by a mismatch between the total supply of resources and the brood's demand for resources (Mock and Parker 1997), high resource availability should reduce the costs of cooperation with siblings, resulting in reduced levels of competitive behaviors. However, despite compelling

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evidence from comparative studies that the evolutionary maintenance of cooperation is influenced by polyandry (Cornwallis et al. 2010; Griffin et al. 2013) and resource availability (Rubenstein and Lovette 2007; Rubenstein 2011; Caro et al. 2016), few studies have investigated facultative adjustments in cooperation in response to these two factors (Falk et al. 2014; Ruch et al. 2014). Such studies are needed to clarify the causal relationship between variation in both female polyandry and resource availability and the expression of conflict versus cooperative behavior at the individual level.

To address this shortcoming, ideally we need a targeted experimental approach. Here, we manipulated polyandry and resource availability in the burying beetle, *Nicorphorus vespilloides*, using a fully factorial design and examined facultative adjustments in levels of conflict within broods, measured as the mean time spent begging by each individual larva in the brood (see Smiseth and Moore 2002; Smiseth et al. 2003). Like all members of the genus *Nicrophorus*, *N. vespilloides* breeds on vertebrate carcasses, which represent a limiting resource shared by the brood (Scott 1998). Parents provision the brood with pre-digested carrion from the carcass, resulting in sibling conflict in the form of competitive begging for access to parental provisioning (Smiseth et al. 2007a,b). Begging behavior is an honest signal of need in *N. vespilloides*, (Smiseth and Moore 2004; Smiseth and Moore 2007) and parental provisioning in response to this begging improves offspring fitness despite the fact that offspring can self-feed from the carcass (Eggert and Muller 1997; Smiseth et al. 2003). Indeed, offspring adjust their begging behavior in response to the number of competitors in the brood, and as a result begging represents a form of scramble competition (Smiseth and Moore 2002; Smiseth et al. 2007a). We also measured the effects of our treatments on female traits thought to influence sibling competition (reviewed in Mock and Parker 1997), specifically 1) reproductive traits (time to laying, clutch size, average egg size, and laying spread) and 2) behavioral traits (percentage of time spent on direct and indirect care). Finally, we measured the effects of our treatments on offspring fitness (number of larvae at dispersal, average individual larval mass at dispersal). Overall, we predicted that there would be increased levels of competitive begging in broods in response to polyandry and/or decreased resource availability.

## METHODS

All beetles used in this study were from an outbred laboratory population maintained at the University of Edinburgh. Beetles were housed individually in clear plastic boxes (124 × 82 × 22 mm) and kept at 20 ± 2 °C under a 16:8 h light:dark cycle. Beetles were fed small pieces of organic beef twice a week. At the start of the experiments beetles were aged 18–27 days post-eclosion.

### Manipulation of relatedness and resource availability

Levels of relatedness between siblings were manipulated by allowing females to mate with either 1 male (monogamous treatment) or 2 males (polyandrous treatment). Pairs of unrelated (to grandparent level) virgin males and females were mated by placing them together in a sealed petri dish. In the polyandrous treatment, the first male was removed from the petri dish after 4 h and replaced with a second male (unrelated at the grandparent level to both the female and her first mate) who was also left to mate with the female for 4 h. In the monogamous treatment, pairs were left together to mate for 8 h. In this treatment, males were briefly removed and reintroduced to their petri dish at the 4 h mark of the mating to control for the disturbance caused when removing the first male in the polyandrous treatment. After mating, females were transferred to a transparent container (170 × 30 × 120 mm, and 60 mm high) filled with 1 cm of moist compost and provided with a previously frozen mouse carcass (supplied from Livefoods Direct Ltd, Sheffield, UK).

We did not genetically assess levels of mixed paternity in the experimental broods. However, we have high confidence that our manipulation of paternity would have resulted in mixed sexed broods. First, our manipulation provided no opportunity for monopolization of paternity by 1 male through pre-copulatory processes. For all replicates in the polyandry treatment (except for 2 which were excluded), both males were observed to mate with the female. In similar experiments on a related species (*N. tomentosus*) where 2 females and 2 males were housed together on a carcass (allowing pre-copulatory processes to occur), paternity was shared in 70% of broods (Scott and Williams 1993). Second, our manipulation provided limited potential for post-copulatory processes (such as sperm competition or female choice) to bias paternity strongly to either of the 2 males. Previous research on this species has shown that sperm precedence does occur but requires high levels of repeated copulations over a 24 h period (Müller and Eggert 1989). In contrast, when 2 males exhibit similar levels of mating, paternity share has been shown to be equal (House et al. 2007). We gave each male only 4 h to mate with the female in the absence of a carcass, and we therefore expect a roughly equal number of copulations and share of paternity between males. Combined with the fact that previous mating experiments with *N. vespilloides* using similar approaches have produced mixed paternity broods (see House et al. 2007; Pettinger et al. 2011; Sakaluk and Müller 2008), this gives us high confidence that our manipulation successfully produced broods of mixed paternity and we interpret our results in line with this.

Resource availability was manipulated by letting females breed and raise offspring on either a small (8–12 g) or a large (21–25 g) mouse carcass. These carcass sizes were chosen based on previous work showing that *N. vespilloides* breeds on carcasses ranging in size from 1 to 40 g (Smiseth and Moore 2002) and that larvae are smaller on carcasses in the lower end of the size range, suggesting an effect of resource limitation on offspring growth (Smiseth et al. 2014). Across all replicates, we removed the male after mating because males are less involved in resource provisioning of offspring than are females and male assistance in provisioning of resources has no detectable effect on offspring growth or survival in laboratory conditions (Smiseth et al. 2005). In total, we had 135 beetle broods: 36 in the monogamous/high resource treatment; 32 in the monogamous/low resource treatment; 34 in the polygamous/high resource treatment; and 33 in the polygamous/low resource treatment.

### Data collection

In *N. vespilloides,* egg laying starts at 21 ± 2 (mean and standard error) h after females are given access to a carcass, and the first eggs of a clutch start hatching 81 ± 3 h after access to a carcass (Smiseth et al. 2006). We collected information on egg laying by placing the boxes on flat-bed scanners (Canon Canoscan 9000F Mark II, Canon Inc., Tokyo, Japan) and scanning the bottom of the breeding boxes every hour until after the completion of oviposition using Vuescan professional edition software (Hamrick Software, Sunny Isles Beach, FL, USA) (Ford and Smiseth 2016). Eggs are visible at the bottom of the breeding box and the visible number of eggs

closely corresponds to the actual clutch size (Monteith et al. 2012). From the scanned images, we counted the number of new eggs laid each hour to determine the laying spread (the time between the first and last egg being laid) and the total number of eggs laid (clutch size). In addition, we measured egg length and width in pixels for 5 randomly chosen eggs in each clutch using the software ImageJ (Abràmoff et al. 2004). The measurements were then converted into metric length (mm) and used to calculate a prolate spheroid volume, $V$, for each egg using the equation $V = (1/6)(\pi w2L)$, where $w$ is the width and $L$ the length of the egg (Berrigan 1991). This data was then used to calculate an average egg size for each clutch. In some instances, the scans produced were of low quality, preventing us from collecting all data on all traits.

Offspring begging peaks 24 h after the first eggs start hatching (Smiseth et al. 2003). We therefore collected behavioral observations of each clutch as close as possible to 24 h after emergence of the first hatched larva (on average, clutches were observed 29 ± 0.4 h after hatching of the first egg). Observations were conducted under photographic red light using instantaneous sampling every 1 min for 30 min in accordance with the protocol previously developed (Smiseth and Moore 2002; Smiseth et al. 2003; Smiseth et al. 2005). To quantify sibling competition, we counted the number of larvae in a given brood that were feeding from the mother and that were begging at each scan. An offspring was scored as feeding when there was mouth-to-mouth contact between it and the mother, and it was scored as begging when raising its head toward the mother while waving the legs when within less than the width of its pronotum (~5 mm) from the mother or touching the mother (Rauter and Moore 1999; Smiseth and Moore 2002). This distance corresponds to the distance from which offspring start begging (Rauter and Moore 1999). We calculated the average percentage of time spent begging by each individual larva in the brood when the female was near the larvae, bi, as $bi = \Sigma b/L \times 100/d$, where $\Sigma b$ is the total number of begging events occurring during the 30 scans in an observation session, $L$ is the brood size at the time of observation, and $d$ is the number of scans during an observation session that the female was within a pronotum width of the offspring (Smiseth et al. 2003; Smiseth and Moore 2004).

We also recorded maternal parental behaviors during these observations (see Walling et al. 2008; Andrews et al. 2017 for similar approaches). Specifically, we estimated the amount of time females spent providing direct care, defined as when the female was provisioning food to the brood (engaging in mouth-to-mouth contact with at least one larva) or consuming carrion (manipulating carrion), versus indirect care, defined as when the female was maintaining the carcass (adding anal or oral secretions to the surface of the carcass, excavating the depression in the soil surrounding the carcass, or moving the carcass from below) or guarding the carcass (standing still in a position where she could defend the brood from predators and conspecifics). All other maternal behaviors that occurred were recorded as non-parental behaviors (self-grooming while on the carcass or being absent from the carcass) and were not analyzed further. We then calculated the percentage of time during the observation period that mothers spent on direct and indirect care, and used these measures in our final analyses of differences in amounts of care between treatments.

Finally, we measured the consequences of our treatments for offspring fitness in terms of offspring survivorship and growth. We measured offspring survivorship by counting the number of larva present in each brood once they had dispersed from the carcass. Dispersal occurs when offspring leave the carcass and settle in the surrounding soil (typically once the carcass has been fully consumed) and is normally synchronous, approximately 144 h after the laying of the first egg in a clutch (Smiseth et al. 2007b). To measure offspring growth, we compared average larval mass between treatments (each larva was weighed individually at dispersal to the nearest 0.001 mg), with initial larval mass (weighed immediately following observations) included as a covariate.

### Statistical analyses

Data were analyzed using Anova (type III) and General Linear Models implemented in R version 3.3.0 (R development core team 2016) through the "aov" and "glm" functions. We ran models examining differences in variables relating to 1) female patterns of reproductive traits (time to laying, clutch size, average egg size, laying spread), 2) social interactions within the family (average percentage of time spent begging by each larva in the brood, percentage of time spent on direct and indirect care by mothers), and 3) offspring fitness (number of larvae at dispersal, average individual larval mass at dispersal). Mating treatment (monogamous vs. polyandrous), resource treatment (low vs. high) and their interaction were entered as fixed factors in each of these models. Clutch size was included as a covariate for models of laying spread and offspring survival, average larval mass at the time of observation was included as a covariate for the model for larval mass at dispersal, and number of larvae at observation was included as a covariate for modelling average percentage of time spent begging. Time elapsed from hatching until the observation was not equal for all broods, but inclusion of this variable as a covariate when analyzing time begging did not affect any model outputs, so was removed from the final model.

All models started with a full set of interactions between dependent variables as well as covariates and we subsequently eliminated non-significant ($P > 0.05$) interaction terms. We report results for models containing all main effects and significant interactions following backward elimination of non-significant interactions. All data were checked for violation of assumptions, and where required, transformed to fit assumptions. The model examining levels of larval begging violated the assumption of normality, due to the presence of an outlier more than two standard deviations away from the mean; after removal of the outlier, the model conformed to assumptions and removal of the outlier did not affect the model's overall interpretation, so results for this model is reported with the outlier removed. Additionally, the model analyzing number of larvae at dispersal was highly overdispersed but corrected by running a negative binomial model.

## RESULTS

### Effects of polyandry and resource availability on female reproductive investment

Of the 135 females mated, 127 successfully laid eggs, with an equal probability of success across treatments (Mating treatment: $\chi_{1,\ 120}$ = -0.75, $P$ = 0.45, Resource treatment: $\chi_{1,\ 120}$ = 0.81, $P$ = 0.42, Mating × Resource: $\chi_{1,\ 119}$ = −0.83, $P$ = 0.41). The average clutch size for the experiment was 26.10 ± 1.43, consistent with findings in other studies (e.g., Müller et al. 1990).

We found no significant differences between monogamous and polyandrous females in the time elapsed between females being placed on the carcass and the laying of their first egg, the number of eggs laid, egg size, or in the laying spread (Table 1). We also

found no effect of resource availability on the time elapsed between females being placed on the carcass and the laying of their first egg (Table 1). We did, however, find a significant effect of the resource treatment on number of eggs laid and on egg size (Table 1). Specifically, females appeared to alter the trade-off between the number and size of eggs depending on resource availability, with a greater number of eggs of smaller size laid in the high resource treatment compared to the low resource treatment (Figure 1). Resource availability also had a significant effect on laying spread, with females laying over a longer time period in the high resource treatment (25.96 ± 1.42 h, $n$ = 54) compared to the low resource treatment (18.92 ± 1.41 h, $n$ = 50) (Table 1).

### Effects of polyandry and resource availability on social interactions within the family

Across all treatments, larvae spent on average 16.78 ± 1.49% of their time begging ($n$ = 78). Mothers spent on average 23.79 ± 1.94% of their time providing direct care ($n$ = 94) and 51.27 ± 2.83% ($n$ = 94) providing indirect care. Overall, we found no significant effect of mating treatment on time spent begging by larvae (Table 2). However, there was a significant interaction effect between resource treatment and brood size on time spent begging by larvae (Table 2). Specifically, there was no effect of brood size on the amount of time larvae spent begging in the high resource treatment, but begging decreased with an increase in brood size in the low resource treatment (Figure 2). We found no effect of mating or resource treatment on the amount of time a female spent on direct and indirect maternal care (Table 2).

### Consequences of polyandry and resource availability for offspring fitness

Across all treatments, the average number of offspring surviving to dispersal was 18.08 ± 0.99, with an individual larval mass of 0.173 ± 0.004 g. We found no effect of the mating treatment on larvae number and size at dispersal (Table 3). Resource treatment also had no effect on larval number at dispersal (Table 3). However, offspring in the high resource treatment had a significantly greater mass at dispersal than offspring in the low resource treatment (Table 3, Figure 3).

## DISCUSSION

Factors that influence the costs and benefits of interacting with family members, such as relatedness between group members and resource availability, are predicted to influence the balance of cooperation and conflict between siblings. We tested this hypothesis by manipulating polyandry and levels of resource availability in a family living beetle with prolonged parent–offspring and sibling–sibling associations. We found no evidence of facultative responses to our

**Table 1**

**Outputs of models examining treatment effects on female reproductive investment**

| Trait | Time taken to lay first egg (h) | Clutch size | Average egg size ($mm^3$) | Laying spread (h) |
|---|---|---|---|---|
| Mating treatment | $F_{1,109}$ = 1.98, $P$ = 0.16 | $F_{1,101}$ = 1.46, $P$ = 0.23 | $F_{1,96}$ = 0.37, $P$ = 0.54 | $F_{1,100}$ = 0.87, $P$ = 0.87 |
| Resource treatment | $F_{1,109}$ = 0.72, $P$ = 0.40 | **$F_{1,101}$ = 9.25, $P$ < 0.01** | **$F_{1,96}$ = 4.37, $P$ = 0.04** | **$F_{1,100}$ = 5.29, $P$ = 0.02** |
| Mating × Resource | $F_{1,108}$ = 0.03, $P$ = 0.86 | $F_{1,100}$ = 0.02, $P$ = 0.88 | $F_{1,95}$ = 0.36, $P$ = 0.55 | $F_{1,97}$ = 0.01, $P$ = 0.93 |
| Clutch size | | | | **$F_{1,100}$ = 25.31, $P$ < 0.01** |
| Resource × clutch size | | | | $F_{1,97}$ = 0.63, $P$ = 0.43 |
| Mating × clutch size | | | | $F_{1,97}$ = 1.30, $P$ = 0.26 |

Significant effects are in bold.

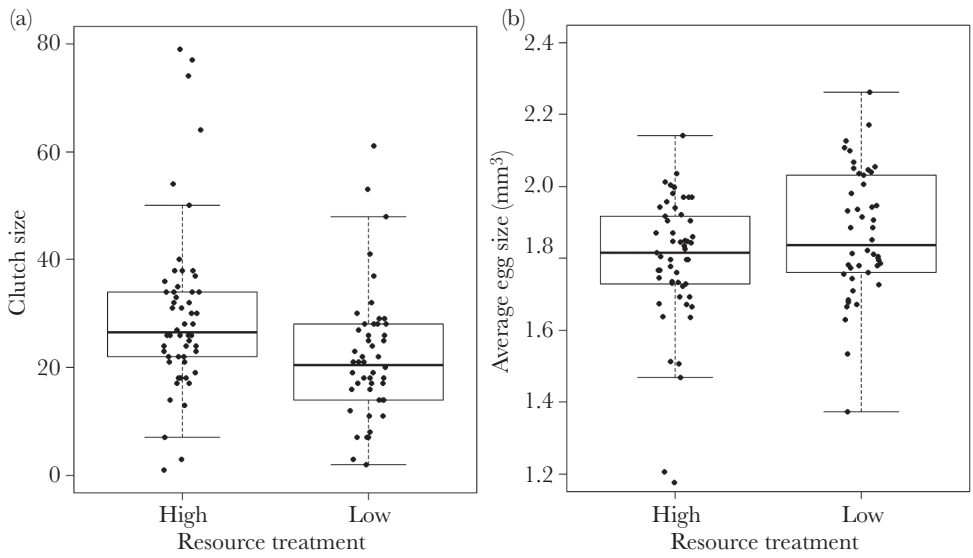


**Figure 1**

Differences between high and low resource availability in female clutch size (a) and average egg size (b). Centre lines represent medians and error bars represent 95% confidence intervals. For (a), $n$ = 54 (high resource treatment) and 50 (low resource treatment); for (b), $n$ = 53 (high resource treatment) and 46 (low resource treatment).

**Table 2**

**Outputs of models examining treatment effects on offspring begging behaviour and direct and indirect forms of female parental behaviour**

| Trait | Time begging (%) | Time spent on direct care (%) | Time spent on indirect care (%) |
|---|---|---|---|
| Mating treatment | $F_{1,72} = 0.09$, $P = 0.77$ | $F_{1,92} = 1.42$, $P = 0.24$ | $F_{1,91} = 1.14$, $P = 0.29$ |
| Resource treatment | **$F_{1,72} = 6.46$, $P = 0.01$** | $F_{1,92} = 1.04$, $P = 0.31$ | $F_{1,91} = 0.45$, $P = 0.50$ |
| Mating × Resource | $F_{1,70} = 0.03$, $P = 0.86$ | $F_{1,90} = 0.27$, $P = 0.60$ | $F_{1,90} = 0.47$, $P = 0.49$ |
| Clutch size | $F_{1,72} = 0.01$, $P = 0.91$ | | |
| Resource × clutch size | **$F_{1,72} = 5.90$, $P = 0.02$** | | |
| Mating × clutch size | $F_{1,70} = 0.13$, $P = 0.72$ | | |

Significant effects are in bold.

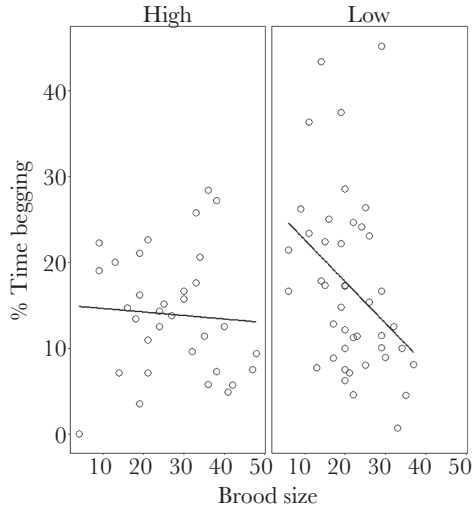


**Figure 2**

Effect of brood size on offspring begging behavior in high and low resource treatments, $n = 33$ (high resource treatment) and 43 (low resource treatment).

**Table 3**

**Outputs of models examining consequences of treatments for offspring survival and growth**

| Trait | Number of larvae at dispersal | Average mass at dispersal |
|---|---|---|
| Mating treatment | $\tilde{\chi}_{1,77} = 0.17$, $P = 0.87$ | **$F_{1,80} = 5.82$, $P = 0.02$** |
| Resource treatment | $\tilde{\chi}_{1,77} = -0.14$, $P = 0.89$ | $F_{1,80} = 0.09$, $P = 0.77$ |
| Mating × Resource | $\tilde{\chi}_{1,74} = 0.30$, $P = 0.76$ | $F_{1,77} = 0.20$, $P = 0.66$ |
| Clutch size | $\tilde{\chi}_{1,77} = 1.43$, $P = 0.15$ | |
| Average mass at observation | | $F_{1,80} = 1.18$, $P = 0.28$ |

Significant results are in bold.

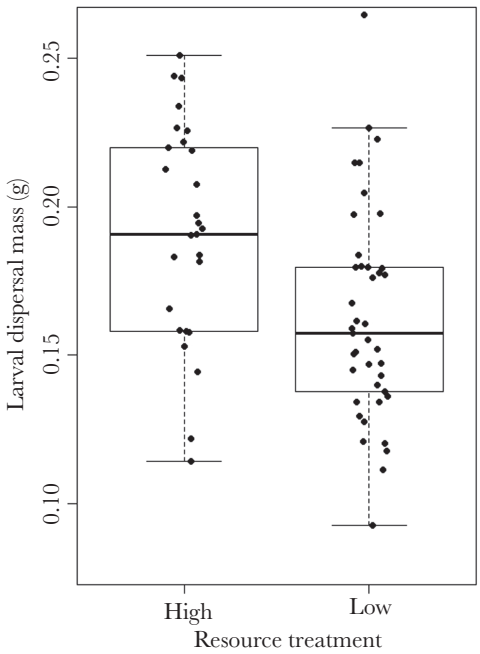


**Figure 3**

Effect of resource treatment on larval mass at dispersal. Centre lines represent group medians and error bars represent 95% confidence intervals, $n = 40$ (high resource treatment) and 48 (low resource treatment).

manipulation of polyandry. However, we did find that females and offspring exhibited facultative responses to resource availability.

## Effects of polyandry and resource availability on female reproductive traits

We found strong effects of resource availability but not female polyandry on our four variables relating to female reproductive output. Specifically, although resource availability had no effect on the timing of onset of egg production, it did influence how females balanced the trade-off between the number and size of eggs, with females on larger carcasses producing more eggs but of a smaller size than females on the smaller carcasses. These results are in line with previous literature on *N. vespilloides* which has shown a reduction in clutch size on carcasses of 10 g or lower (Müller et al. 1990). While we were unable to examine whether this resulted in equivalent investment overall (because we did not measure total brood mass at the egg stage), these results suggest that resource availability influences pre-laying reproductive decisions by the mother.

We also found that females increased their laying spread when breeding on larger carcasses. As laying spread corresponds with hatching spread in burying beetles (Smiseth et al. 2006), this potentially suggests an adaptive adjustment in hatching spread in response to resource availability, as has been suggested for hatching asynchrony in birds (e.g. Wiebe 1995; Mock and Parker 1997). Increased hatching asynchrony in burying beetles increases asymmetric competitive abilities among the brood, with later hatched offspring begging more but growing less than earlier hatched offspring (Smiseth et al. 2008). This may facilitate adaptive brood reduction under stressful environmental conditions (Lack 1947). However, our results were in the opposite direction to those predicted under the brood reduction hypothesis (laying spread was greatest when resource

availability was high). There are several potential explanations for these contrasting results. First, resource availability at the time of provisioning is known as carcasses are obtained prior to egg laying. Thus, females could manipulate brood size by adjusting egg number rather than through hatching asynchrony. Second, if females were to manipulate brood size post laying, the most direct and energetically efficient way to achieve this is through filial cannibalism rather than through establishing asymmetric sibling hierarchies (Bartlett 1987; Müller et al. 1990). While we still do not have an explanation for why females might increase laying spread on larger carcasses, these results do highlight the need for continued work on the environmental triggers and consequences for variation in laying and hatching spread within this system.

## Effects of polyandry and resource availability on social interactions within the family

We found no effects of female polyandry on intra-brood conflict (measured as mean time spent begging by individual larvae in a brood). This suggests that, in contrast to theoretical predictions (i.e., Hamilton's rule; Hamilton 1964; see also Briskie et al. 1994), female polyandry does not influence intra-brood conflict in *N. vespilloides*. There are several potential explanations for the lack of facultative responses observed here. First, our manipulation of polyandry may not have resulted in a decrease in relatedness between brood mates, due to post-copulatory processes that bias paternity towards a single male. However, we argue that this is unlikely given the nature of our study organism and experimental manipulation (see Methods for further justification). Alternatively, relatedness may have been decreased but offspring may lack the relevant mechanisms to assess kin and respond accordingly. While offspring recognition of adults has been shown to occur in burying beetles (Mäenpää et al. 2015), we know little about the extent of sibling-sibling recognition. Indeed, depending on the frequency of mixed paternity broods in the wild (which is currently unknown; but see Müller and Eggert 1989), there may have been limited scope for selection on kin discrimination (Cornwallis et al. 2009; Cornwallis et al. 2010). Alternatively, selection on kin in offspring may have been outweighed by selection on mothers to prevent the expression of father-specific chemical signatures in offspring (as suggested to occur in another family living insect, the European earwig; Meunier and Kölliker 2012; Wong et al. 2014). If so, this would prevent the evolution of offspring kin recognition, thereby minimizing costly sibling conflict and maximizing fitness from the mother's perspective. While these explanations remain largely speculative at present, *N. vespilloides* offers an interesting system to study the role that levels of polyandry have on the emergence of recognition behavior using experimental evolution approaches.

In contrast to polyandry, we found evidence that resource availability does influence levels of intra-brood conflict, with larvae spending more time begging on smaller mice carcasses compared to larger ones. Interestingly, this was only observed in smaller broods. This may appear counter-intuitive, as levels of competition should be relatively low in small broods compared to large broods. A potential explanation for these results is that *N. vespilloides* offspring are only partially dependent on parents for food because they can self-feed directly from the carcass (Smiseth et al. 2003; Capodeanu-Nägler et al. 2016). Thus, at higher brood sizes, there may be a limit to the amount of provisioning the mother can provide to offspring, thereby reducing the effectiveness of begging and leading to more self-feeding behavior by offspring (Smiseth et al. 2007a). This argument mirrors Trumbo's (1992) explanation for between-species patterns of larvae dependence on parental feeding: in species that rear large broods, larvae are selected to maintain their independence for feeding, because parents cannot attend to each larva as well as parents in species where brood sizes are smaller. This could potentially be tested in the future by observing levels of offspring begging in response to manipulating brood size on a fixed carcass size (i.e. altering offspring density). An alternative explanation is that begging behavior may act as cooperative rather than a competitive behavior, functioning to solicit maximum levels of overall parental care when resources are limited as has been observed in the black-head gull (Mathevon and Charrier 2004) and the banded mongoose (Bell 2007). Indeed, a recent study on *N. vespilloides* found some evidence of sibling cooperation in the absence of caring parents (Schrader et al. 2015). However, given that begging only occurs in the presence of caring parents (Rauter and Moore 1999; Smiseth and Moore, 2002), there is no evidence that begging per se is cooperative in this species.

## Consequences of polyandry and resource availability for offspring fitness

Given our finding that polyandry had no effect on either female reproductive traits or intra-brood competition (which could decrease offspring fitness), it is not surprising that we did not observe effects of polyandry on offspring fitness. In contrast, we found that resource availability influenced offspring growth but not survival, with offspring raised on larger carcasses growing to a larger size than those on small carcasses. Our finding of higher growth on larger carcasses is intuitive and matches previous findings in this and other species of *Nicrophorus* (Bartlett and Ashworth 1988; Scott and Traniello 1990; Trumbo 1992; Eggert and Muller 1997). However, previous work on *N. vespilloides* found lower survival on smaller carcasses (Smiseth et al. 2008), which contrasts with our result of no effect of carcass size on larval survival. This discrepancy may arise from the smaller carcass size used in the previous study (5 g in Smiseth et al. 2008 vs. 8–12 g in this study) if there is a non-linear relationship between carcass size and larval survival. For example, the discrepancy might occur due to a threshold in carcass size between 5–8 g at which decreasing carcass size negatively impacts larval survival. However, further empirical tests are required to confirm whether this is the case.

## Conclusions

Here, we tested for facultative adjustments of mothers and their offspring to changes in resource availability and polyandry to gain insights into the role of these factors on family living. We found that, under restricted resource availability, there was reduced begging in larger broods, indicating the potential for sustained changes in resource availability to lead to evolutionary change in family dynamics. By contrast, we found no responses of family members to polyandry. Despite finding no facultative response of mothers or their offspring to polyandry, it is important to note that polyandry could still lead to responses over evolutionary timescales. To detect such responses in future studies will necessitate the use of comparative and/or experimental evolution approaches.

## SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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Data accessibility: Analyses reported in this article can be reproduced using the data provided by Botterill-James et al. (2017).

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